OIPE JC98 MAY 13 2005 PATENT & TRADEMARK OFFICE

Atty. Dkt. No. 057491-0413

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Desmond MASCARENHAS

Title: NULL IGF FOR THE TREATMENT OF CANCER

Appl. No.: 09/399,120

Filing Date: 09/20/1999

Examiner: A. Gupta

Art Unit: 1654

TRANSMITTAL OF BRIEF ON APPEAL

Mail Stop APPEAL BRIEF-PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith is an amendment in the above-identified application.

[X] Small Entity status under 37 C.F.R. § 1.9 and § 1.27 has been established by a previous assertion of Small Entity status.

[X] Brief on Appeal.

[X] A check in the amount of $475.00 is enclosed in payment of fee for filing a brief in support of an appeal under 37 CFR 1.17(c) and a petition for an extension of time under 37 C.F.R. § 1.136(a).

[X]	Extension for response filed within the second month:	$450.00
[X]	Brief on Appeal:	$500.00
[X]	Total Fee:	$950.00

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[X]	Small Entity Fees Apply (subtract ½ of above):	$475.00

[X] A check in the amount of $475.00 is enclosed in payment of fee for filing a brief in support of an appeal under 37 CFR 1.17(c).

[ ] Please charge Deposit Account No. 19-0741 in the amount of $00.00.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date May 13, 2005

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OIPE JC98
MAY 13 2005
PATENT & TRADEMARK OFFICE

Attorney Docket No. 057491-0413

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF APPEALS AND PATENT INTERFERENCES

Applicant: Desmond MASCARENHAS

Title: NULL IGF FOR THE TREATMENT OF CANCER

Appl. No.: 09/399,120

Filing Date: 9/20/99

Examiner: Anish GUPTA

Art Unit: 1654

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
P.O. Box 1450
Alexandria, VA 2231301450

Sir:

This Appeal Brief is being filed with a check in the amount of $475.00 covering the appeal and extension fees. If this amount is deemed insufficient, Appellants authorize charging any deficiency (as well as crediting any balance) to deposit account 19-0741.

This is an appeal from a final Office Action dated August 13, 2004, finally rejecting claims 1-10, 16 and 18-50 under 35 U.S.C. § 112, first paragraph, as allegedly not enabled.

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I. REAL PARTY IN INTEREST

The real party in interest is the assignee, CELTRIX PHARMACEUTICALS, INC., 800 Leigh Street, Richmond, VA 23219.

II. RELATED APPEALS AND INTERFERENCES

Neither the appellant, appellant's legal representative, or their assignee is aware of any related appeals or interferences that will affect directly or be affected directly by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

All pending claims, *i.e.*, claims 1-10, 16 and 18-50, are on appeal and are attached hereto as APPENDIX A.

IV. STATUS OF AMENDMENTS

In response to a restriction requirement dated August 17, 2001, claims 1-10 and 16 were elected for prosecution on the merits and claims 11-15 and 17 were withdrawn from consideration. In response to a non-final Office Action dated September 19, 2001, claim 1 was amended, and in response to a non-final Office Action dated May 21, 2002, claims 7-8 were amended and new claims 18-44 were added.

In response to a November 5, 2002 final Office Action, claims 11-15 and 17 were cancelled and new claims 45-46 were added. An Advisory Action that issued on June 3, 2003, indicated that Appellants April 7, 2003 amendment was not entered and therefore, only claims 1-10, 16 and 18-44 were under examination. Subsequently, an RCE was filed and new claims 45-47 were added.

In response to a non-final office action dated September 24, 2003, Appellants amended claims 45-46 and added new claims 48-50. In a final Office Action dated August 13, 2004, claims 1-10, 16 and 18-50 were under examination and remained finally rejected.

V. SUMMARY OF THE CLAIMED INVENTION

The claimed invention provides methods for slowing the growth rate of a tumor and methods for slowing the progression of a cancer. The methods comprise administering a null insulin-like growth factor-I (IGF-I) to a subject having cancer. A null IGF-I, as described in the present application, refers to IGF-I which: (1) has amino acid sequence alterations at one or more sites in the molecule, (2) retains its ability to bind IGFBP-3, and (3) is altered in its receptor binding and/or activating properties (*e.g.*, has little or no binding to the type I IGF receptor but can bind the type II IGF receptor, or has substantially reduced binding to both type I and II receptors) (specification at 5, lines 17-23). In addition, the specification provides guidance as to specific null IGF-Is that can be employed in the methods of the claimed invention (specification at 5, lines 26-31 and at 7, lines 3-6).

Appellants have surprisingly found that a null IGF-I, when administered uncomplexed to IGFBP-3, is effective in alleviating the symptoms of cancer. In contrast, administration of a null IGF-I/IGFBP-3 complex is ineffective (specification at 5, lines 2-9). This finding was unexpected because IGFBP-3 was known to substantially increase the half-life of IGF-I and to increase the efficacy of IGF-I (*Id.*). Thus, it was expected that a null IGF-I/IGFBP-3 complex would be more efficacious at alleviating the symptoms of cancer than administration of IGF-I alone.

As claimed in the present invention, an uncomplexed null IGF-I is administered to a subject to slow the growth rate of a tumor or the progression of a cancer (specification at 6, lines 17-21). The null IGF-I of the present invention can be used to treat any cancer, including breast, prostate and lung cancer (specification at 7, lines 1-2). The specification also provides guidance on modes of administration and dosage of the null IGF-I (specification at 8, lines 1-21).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The Examiner maintained a rejection of claims 1-10, 16, and 18-50 under U.S.C. § 112, 1st paragraph, as allegedly not enabled by the specification.

VII. ARGUMENTS

A. Rejection of Claims 1-10, 16 and 18-50 Under 35 U.S.C. § 112, first paragraph

1. Examiner's Basis for the Enablement Rejection

The Examiner rejected claims 1-10, 16 and 18-50 as allegedly not enabled, asserting that the ordinary meaning of "null IGF" is unclear, that it is unclear as to what modifications in IGF would render an IGF a null IGF (September 24, 2003 Office Action at 3), and that "null IGF" is not a specific class of compounds (August 13, 2004 Office Action at 3). Moreover, the Examiner indicated that "one of ordinary skill in the [art] does not readily know which modification would lead to the desired functional activity" and that "the functional definition [of null IGF] does not provide any guidance as to the structural definition of the [n]ull IGF" (September 24, 2003 Office Action at 4).

Furthermore, the Examiner stated that "the instant application has not provided ample guidance for one to conclude that the single example of Y60L bears a reasonable correlation to the entire scope of the claim" (August 13, 2004 Office Action at 3).

Continuing, the Examiner rejected all pending claims because "rodents are better predictors of human reaction to cardiovascular or anti-inflammatory agents than cancer..." (September 24, 2003 Office Action at 4) and that "cancer animal models and cell models, although provide valuable information for delivery of therapeutics, *do not correlate to human in-vivo efficacy*" (*Id.* at 5). In other words, the Examiner believed that "animal models are ineffective in predicting cancer" (*Id.* at 4).

Along these lines, the Examiner additionally indicated that Appellant's previously submitted references to support the use of PC-3 xenograft animal models as a model for prostate cancer "provide[] very little evidence to rebut the assertion . . . [that] other types of cancer beyond prostate tumors [are enabled]" (August 13, 2004 Office Action at 4).

2. The Ordinary Meaning of Null IGF is Understood by a Skilled Artisan

References describing the state of the art regarding null IGF at the time of filing were submitted with Appellant's April 7, 2003 response and are provided herewith (Attached as APPENDIX B). These citations indicate that null IGFs were well defined at the time of filing. In addition, a person of skill in the art would know which amino acids could be changed based on these references and the regions identified as "critical" for IGF receptor and IGFBP binding (*See*, for example, Jannson *et al.*, *Biochemistry*, 36:4108-17 (1997), previously submitted with Appellant's August 21, 2002 response and provided herewith in APPENDIX B).

Furthermore, the specification describes that null IGFs of the claimed invention have a reduced affinity for the IGF receptor and a maintained ability to complex IGFBP-3. Exemplary null IGFs are also outlined on page 5 and example 2 of the application.

Moreover, the specification describes precise modifications that can be made to an IGF molecule to render it "null," and discloses references which illustrate null IGFs that are suitable for use in the claimed invention (*see* specification at page 7). In addition, many of the claims are directed to a defined subset of null IGF molecules and, in fact, recite specific amino acid substitutions. Therefore, not only are Appellant's claims drawn to methods of using null IGF compounds, which, as discussed above, possess certain functional features, many of the pending claims further recite structural features of a null IGF.

3. Claims Drawn to Methods Comprising Administering a Null IGF are Enabled

The present application claims methods for slowing the growth rate of a tumor or slowing progression of a cancer comprising administering a null IGF-I. Null IGFs were well defined at the time of filing and one of skill in the art would know how to make and use a null IGF in view of the literature and present specification.

Additionally, the present disclosure and accompanying declaration (previously submitted with Appellant's April 7, 2003 response and provided herewith in APPENDIX C) provides that null IGFs have a lower affinity for an IGF-I receptor but are still able to bind the major IGFBP-3 binding protein at least as well as wild-type IGF-I. Thus, it would be

expected that any null IGF-I that satisfies these properties can be used to slow wild-type IGF-I induced tumor cell growth (specification at 6, lines 19-25 and declaration at paragraphs 4 and 5).

Moreover, the present application offers numerous examples of null IGFs for use in the claimed invention. For example, the specification provides citations describing null IGFs and recites that null IGFs include those

> in which one or more IGF-I's tyrosine residues (i.e., residues 24, 31, or 60) are replaced with non-aromatic residues (i.e., other than tyrosine, phenylalanine or tryptophan), variants where amino acid residues 49, 50, 51, 53, 55 and 56 are altered (for example, where residues 49-50 are altered to Thr-Ser-Ile or where residues 55-56 are altered to Tyr-Gln), and combinations thereof.

Specification at 5, lines 26-31. These null IGFs are also specifically claimed (*see, for example*, pending claims 7-9, 19-21, and 45-46).

Therefore, contrary to the Examiner's assertions, it would not be "unclear as to what modifications in IGF would render an IGF a null IGF" or that "one of ordinary skill in the [art] does not readily know which modification would lead to the desired functional activity" in view of the teachings in the art and instant specification. Appellant's disclosure, in combination with knowledge known in the art at the time the application was filed, enables one of skill in the art to practice the claimed invention.

4. Claims Drawn to Methods Comprising a Null IGF With a Substitution at Position 60 are Enabled

Claims 7, 18, 26 and 47-50 are directed to methods for slowing the growth rate of a tumor or slowing progression of a cancer comprising administering a null IGF-I, wherein the residue at position 60 of the null IGF-I is altered to a non-aromatic residue (claims 7 and 48) and more specifically, to a leucine residue (Y60L) (claims 18 and 49). These null IGF-I compounds are further claimed in the methods specifically directed to the treatment of prostate cancer (claims 26, 47 and 50).

The working examples in the specification provide that a Y60L null IGF was used to treat an animal model for human prostate cancer. Results indicated that mice treated with the

Y60L null IGF "had a substantially greater survival" (Example 2 of the specification at 11-12). Therefore, at least the claims drawn to Appellant's working examples are enabled by the specification.

Indeed, according to the PTO's own rules, "[a]s long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement...is satisfied" (M.P.E.P. § 2164.01, *citing In re Fisher*, 427 F.2d 833 (CCPA 1970)). As Example 2 of the specification specifically describes the use of a Y60L null IGF for the treatment of prostate cancer *in vivo*, Appellant has fully complied with the enablement requirement of 35 U.S.C. § 112, first paragraph.

With regard to claims drawn to methods comprising administering a null IGF compound, wherein position 60 of the null IGF-I contains a non-aromatic residue substitution, one of skill in the art would expect that replacing this replacement would also disrupt IGF receptor binding. Since tyrosine at position 60 is needed to preserve normal IGF-I binding to the type 1 and type 2 receptors, changing this residue will alter normal IGF-I activity. In fact, Bayne teaches that Tyr^{60} is "involved in the high affinity binding of IGF-I to the type 1 IGF receptor...[and] for maintaining binding to the type 2 IGF receptor" (Bayne *et al., J. Biol. Chem.*, 265:15648-15652 (1990), previously submitted with Appellant's April 7, 2003 response and provided herewith in APPENDIX B). Bayne further teaches that Tyr^{60} of IGF-I is analogous to Tyr^{A19} of insulin, and that mutations in A19 "result in dramatic loss of insulin receptor binding" (*id.* at 15651). The reference further goes on to describe that the loss of interaction between Tyr^{60} and Ile^{43} of insulin, as well as moderate conformational changes in the receptor binding region, will affect IGF receptor binding (*id.*).

Therefore, for at least these reasons, claims directed to methods comprising administering a null IGF, wherein a residue at position 60 of the null IGF is substituted with a lysine residue or another non-aromatic residue, are enabled. Claims drawn to using these null IGFs for the treatment of prostate cancer are enabled as well.

5. Methods for Treating Prostate Cancer are Enabled by the Specification

The working examples of the present invention utilize an *in vivo* animal model for prostate cancer. Specifically, the specification describes that nude mice were implanted with human prostate PC-3 tumor xenografts (Example 2 of the specification at 11-12) and administered a null IGF after tumor growth. Claims 4, 26, 30, 34, 38, 42, 47 and 50 are directed to methods for slowing the growth rate of a tumor or slowing progression of a cancer comprising administering a null IGF-I to a subject having prostate cancer.

As summarized in the Examiner's Basis for the Enablement Rejection section above, the Examiner failed to appreciate the value of animal models in general, and xenograft models in particular, and used this as a basis for doubting the correlation between Appellant's *in vivo* results and a human therapeutic effect of null IGFs. While it is understood that no animal model is 100% predictive of the human condition, one cannot correctly conclude that efficacy of a therapeutic agent in an animal model bears zero correlation with therapeutic efficacy in a person. Certainly, none of the references cited by the Examiner to date indicate that there is no association between the pathogenesis of cancer in an animal model and a human.

Moreover, there is no per se requirement that an animal model used in a patent application to support a claim of biological activity must be demonstrated to be "art-recognized". Unless there is a specific reason to doubt the correlation between a certain experimental animal and treatment of the disease condition itself, the evidence should be accepted as presumptively accurate (*In re Marzocchi*, 169 U.S.P.Q. 367, 369 (CCPA 1971). For example, in *In re Langer*, 183 U.S.P.Q. 288 (CCPA 1974), the Court held that the evidence required to rebut a case of non-enablement or lack of utility established by the Examiner need only be sufficient to rebut the evidence presented by the Examiner. Thus, in *Langer*, 183 U.S.P.Q. at 297, applicants' evidence from an animal model, whether or not the animal model was "art-recognized", was more than sufficient to rebut the doubts raised by the in vitro data provided in the references cited by the Examiner.

Accordingly, it is sufficient to use a "standard experimental animal," which was defined *in In re Krimmel,* 130 U.S.P.Q. 215, 219 (1961), as "whatever animal is usually used by those

skilled in the art to establish the particular pharmaceutical application in question." See also, *In re Hartop*, 135 U.S.P.Q. 419 (1962). To support the sufficiency of Appellant's mouse model, appended hereto in APPENDIX D are copies of abstracts (previously submitted with Appellant's April 7, 2003 response) demonstrating that a PC-3 animal model is frequently used by one of skill in the art to determine the therapeutic utility of a particular drug. Also, the declaration filed concurrently herewith attests to the relevance of the animal model used in the claimed invention ("as predicted from studies in the PC-3 animal model, a null IGF applied to any cancer associated with IGF-induced cellular proliferation would have an anti-cancer effect" (declaration at paragraph 8)).

6. Methods for Treating Cancers Other Than Prostate Cancers are Enabled by the Specification

As indicated in the appended declaration, the null IGF technology of the claimed invention is suitable for treatment of a human cancer and is not limited to prostate cancer. See, declaration at paragraph 7. Although the PC-3 animal model is a prostate cancer model, the therapeutic utility of Y60L in the PC-3 xenograft can be utilized to support the utility of a null IGF in other cancers. Indeed, the relationship between IGFs and cancer risk has been well studied and null IGFs are therefore suitable for slowing the growth rate and/or progression of any cancer for which IGFs have been implicated. Abstracts implicating IGFs in cancer progression were previously submitted with Appellant's April 7, 2003 and August 21, 2002 responses and are provided herewith in APPENDIX E.

* * *

Because the specification describes how to make and use the claimed invention, Appellants courteously request that the Board reverse the Examiner's rejection of the claims.

VIII. EVIDENCE

Copies of any evidence entered and relied upon in the appeal is submitted herewith in APPENDICES B-E.

APPENDIX B

Baxter *et al.*, *J. Biol. Chem.*, 267:60-5 (1992)

Bayne *et al.*, *J. Biol. Chem.*, 264:11004-08 (1988)
Bayne *et al.*, *J. Biol. Chem.*, 265:15648-52 (1990)
Jannson *et al.*, *Biochemistry*, 36:4108-17 (1997)
Loddick *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 95:1894-98 (1998)
Martin *et al.*, *Endocrinology*, 131:1703-10 (1992)
Smith *et al.*, *Endocrinology*, 131:2733-41 (1992)

APPENDIX C

1.132 Declaration of Andreas Sommer

APPENDIX D

An *et al.*, *Prostate*, 34:169-74 (1998)
Jungwirth *et al.*, *Prostate*, 32:164-72 (1997)
Jungwirth *et al.*, *Br. J. Cancer*, 75:1585-92 (1997)
Lamharzi *et al.*, *Regul. Pept.*, 77:185-92 (1998)
Pinski *et al.*, *Int. J. Cancer*, 55:963-7 (1993)
Plonowski *et al.*, *Prostate*, 52:173-82 (2002)
Plonowski *et al.*, *Int. J. Cancer*, 98:624-9 (2002)
Plonowski *et al.*, *Prostate*, 44:172-80 (2000)
Rubenstein *et al.*, *J. Surg. Oncol.*, 62:194-200 (1996)
Rubio *et al.*, *Prostate*, 44:133-43 (2000)
Rubio *et al.*, *Lab. Invest.*, 78:1315-25 (1998)
Seki *et al.*, *Hum. Gene Ther.*, 13:761-73 (2002)
Shevrin *et al.*, *Prostate*, 15:187-94 (1989)
Soos *et al.*, *Anticancer Res.*, 17:4253-8 (1997)
Ware *et al.*, *J. Urol.*, 128:1064-7 (1982)
Waters *et al.*, *Prostate*, 26:227-34 (1995)
Wilson *et al.*, *Cell Mol. Biol. Res.*, 39:751-60 (1993)

APPENDIX E

Adenis *et al.*, *Eur. J. Cancer*, 31A:50-5 (1995)
Akagi *et al.*, *Cancer Res.*, 58:4008-14 (1998)
Ankrapp *et al.*, *Cancer Res.*, 53:3399-404 (1993)
de Cupis *et al.*, *Br. J. Pharmacol.*, 120:537-43 (1997)
Furstenberger *et al.*, *Lancet Oncol.*, 3:298-302 (2002)
Garrouste *et al.*, *Endocrinology*, 138:2021-32 (1997)
Gebauer *et al.*, *Anticancer Res.*, 18:1191-5 (1998)
Guo *et al.*, *J. Cell Physiol*, 175:141-8 (1998)
Hankinson *et al.*, *Lancet*, 351:1393-6 (1998)
Helle *et al.*, *Acta Oncol.*, 25 Suppl. 5:19-22 (1996)
Jernstrom *et al.*, *Eur. J. Cancer Prev.*, 6:330-40 (1997)
Kawamura *et al.*, *Anticancer Res.*, 14:427-31 (1994)
Lee *et al.*, *J. Endocrinol.*, 152:39-47 (1997)
Macaulay *et al.*, *Eur. J. Cancer Clin. Oncol.*, 24:1241-2 (1998)

Macauly *et al.*, *Br. J. Cancer*, 57:91-3 (1988)
Manousos *et al.*, *Int. J. Cancer*, 83:15-7 (1999)
Mazzoccoli *et al.*, *Anticancer Res.*, 19:1397-9 (1999)
Michell *et al.*, *Growth Factors*, 14:269-77 (1997)
Minuto *et al.*, *Cancer Res.*, 46:985-8 (1986)
Mishra *et al.*, *Growth Horm. IGF Res.*, 8:473-9 (1998)
Moschos *et al.*, *Oncology*, 63:317-32 (2002)
Pollak, M., *Eur. J. Cancer*, 36:1224-8 (2000)
Pollak, M., *Recent Results Cancer Res.*, 152:63-70 (1998)
Rubini *et al.*, *Exp. Cell Res.*, 251:22-32 (1999)
Tisi *et al.*, *Int. J. Biol. Markers*, 6:99-102 (1991)
Vadgama *et al.*, *Oncology*, 57:330-40 (1999)
Vink-van Wijngaarden *et al.*, *Eur. J. Cancer*, 32A:842-8 (1996)
Wang *et al.*, *Curr. Cancer Drug Targets*, 2:191-207 (2002)
Werner *et al.*, *Mol., Genet. Metab.*, 71:315-20 (2000)
Xie *et al.*, *Eur. J. Cancer*, 1717-23 (1999)
Yu *et al.*, *J. Natl. Cancer Inst.*, 91:151-6 (1999)
Yu *et al.*, *J. Natl. Cancer Inst.*, 92:1472-89 (2000)
Zarrilli *et al.*, *Cell Growth Differ.*, 5:1085-91 (1994)

IX. CONCLUSION

The Board is respectfully requested to reconsider and reverse the outstanding rejection.

Respectfully submitted,

Date: 5-13-05

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APPENDIX A: CLAIMS ON APPEAL

1. A method for slowing the growth rate of a tumor, comprising: administering an effective amount of uncomplexed null insulin-like growth factor I (IGF-I) to a subject having cancer.

2. The method of claim 1, wherein said cancer is selected from the group consisting of breast, prostate, colon and lung cancer.

3. The method of claim 2, wherein said cancer is breast cancer.

4. The method of claim 2, wherein said cancer is prostate cancer.

5. The method of claim 2, wherein said cancer is colon cancer.

6. The method of claim 2, wherein said cancer is lung cancer.

7. The method of claim 1, wherein the residue at position 60 of the amino acid sequence of said null IGF-I is altered to a non-aromatic residue.

8. The method of claim 7, wherein the residue at position 24 or 31 of said amino acid sequence of said null IGF-I is additionally altered to a non-aromatic residue.

9. The method of claim 7, wherein said null IGF-I is additionally altered at a position selected from the group of positions 49, 50, 51, 53, 55 and 56.

10. The method of claim 1, wherein said null IGF-I is administered at about 0.01 to about 50 milligrams per kilogram total body weight per day (mg/kg/day).

16. A method for slowing progression of a cancer comprising: administering an effective amount of uncomplexed null insulin-like growth factor I (IGF-I) to a subject having cancer, thereby slowing progression of the cancer.

18. The method of claim 1, wherein the residue at position 60 of the amino acid sequence of said null IGF-I is altered to a leucine residue.

19. The method of claim 1, wherein the residue at position 24 of the amino acid sequence of said null IGF-I is a non-aromatic residue.

20. The method of claim 19, wherein the residue at position 31 of said amino acid sequence of said null IGF-I is a non-aromatic residue.

21. The method of claim 1, wherein the residues at positions of 24, 31 and 60 of the amino acid sequence of said null IGF-I are altered to a non-aromatic residue.

22. The method of claim 1, wherein the amino acid sequence of said null IGF-I is altered such that residues 28 to 37 are replaced with four glycine residues.

23. The method of claim 22, wherein the residue at position 60 of the amino acid sequence of said null IGF-I is a non-aromatic residue.

24. The method of claim 22, wherein the residue at position 24 of the amino acid sequence of said null IGF-I is a non-aromatic residue.

25. The method of claim 7, wherein said cancer is breast cancer.

26. The method of claim 7, wherein said cancer is prostate cancer.

27. The method of claim 7, wherein said cancer is colon cancer.

28. The method of claim 7, wherein said cancer is lung cancer.

29. The method of claim 8, wherein said cancer is breast cancer.

30. The method of claim 8, wherein said cancer is prostate cancer.

31. The method of claim 8, wherein said cancer is colon cancer.

32. The method of claim 8, wherein said cancer is lung cancer.

33. The method of claim 19, wherein said cancer is breast cancer.

34. The method of claim 19, wherein said cancer is prostate cancer.

35. The method of claim 19, wherein said cancer is colon cancer.

36. The method of claim 19, wherein said cancer is lung cancer.

37. The method of claim 20, wherein said cancer is breast cancer.

38. The method of claim 20, wherein said cancer is prostate cancer.

39. The method of claim 20, wherein said cancer is colon cancer.

40. The method of claim 20, wherein said cancer is lung cancer.

41. The method of claim 21, wherein said cancer is breast cancer.

42 The method of claim 21, wherein said cancer is prostate cancer.

43. The method of claim 21, wherein said cancer is colon cancer.

44. The method of claim 21, wherein said cancer is lung cancer.

45. The method of claim 1, wherein said null IGF-I is selected from the group consisting of [Leu 60] IGF-I, [Ala31, Leu60] IGF-I; [Leu24, Leu60] IGF-I; [Leu24, Ala31, Leu60] IGF-I; [1-27, Gly4, 38-70] IGF-I; [Ser24] IGF-I; and [Leu24, 1-62] IGF-I.

46. The method of claim 4, wherein said null IGF-I is selected from the group consisting of [Leu 60] IGF-I, [Ala31, Leu60] IGF-I; [Leu24, Leu60] IGF-I; [Leu24, Ala31, Leu60] IGF-I; [1-27, Gly4, 38-70] IGF-I; [Ser24] IGF-I; and [Leu24, 1-62] IGF-I.

47. The method of claim 18, wherein said cancer is prostate cancer.

48. The method of claim 16, wherein the residue at position 60 of the amino acid sequence of said null IGF-I is altered to a non-aromatic residue.

49. The method of claim 48, wherein said non-aromatic residue is a leucine residue.

50. The method of claim 48 or 49, wherein said cancer is prostate cancer.

Structural Changes in Insulin-like Growth Factor (IGF) I Mutant Proteins Affecting Binding Kinetic Rates to IGF Binding Protein 1 and IGF-I Receptor

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ABSTRACT: Ligand binding properties of five single amino acid substituted variants (V11A, D12A, Q15A, Q15E, and F16A) of human insulin-like growth factor I (IGF-I) were analyzed with respect to their binding affinities and binding kinetics to recombinant IGF binding protein 1 (IGFBP-1) and a soluble form of the IGF type I receptor (sIGF-I^R), respectively. Side chains of the substituted residues are all predicted to be the most surface exposed in the α-helical portion of the B-region of the IGF-I molecule. The IGF-I variants were produced as fusion proteins to a IgG(Fc) binding protein domain, Z. Ligand binding kinetic rates were determined using BIAcore biosensor interaction analysis technology. All IGF-I variants showed altered binding affinities to both IGFBP-1 and sIGF-I^R. Secondary structure content of the IGF-I variants was estimated using far-UV circular dichroism spectroscopy, followed by variable selection secondary structure calculations. The amount of calculated α-helicity is reduced for all the mutants, most predominantly for IGF-I(V11A) and IGF-I(F16A) proteins. Surprisingly, most of the effects of reduced binding affinities to both target proteins are attributed to lowered on-rates of binding, and these are correlated with the amount of α-helicity in each IGF-I variant. In addition, in some of the IGF-I variants, lowered off-rates of binding are observed. From the results, we propose that IGF-I is unusually sensitive to structural changes by surface amino acid substitutions in the B-region of the molecule. Therefore, biochemical or biological properties of amino acid substituted variants of IGF-I cannot be used in a straightforward way to dissect the direct involvement in binding of individual amino acid residues since structural changes may be involved.

Human insulin-like growth factor I (IGF-I)[1] is a single chain 70 amino acid growth factor with structural homology to proinsulin. The early evolutionary origin of the IGF-like peptides and the highly conserved amino acid sequences among vertebrate species emphasize the important role of IGF-I (LeRoith et al., 1993). IGF-I displays a complex interaction scheme, as the hormone has a key function in the regulation of cellular proliferation and differentiation. The potency of IGF-I is regulated through the interaction with six homologous circulating serum binding proteins, IGFBP-1 to IGFBP-6 (Rechler, 1993). Cellular responses of IGF-I are mediated through the IGF type I receptor (IGF-I^R) tyrosine kinase activity (Morgan et al., 1986) and potentially also through membrane-associated IGFBPs (Oh et al., 1993a). The molecular interactions of all these molecules have been extensively studied during the past decade, yet there is no structural information from crystallography or NMR spectroscopy available of any of the complexes. The different binding specificities of the IGF-I molecule have a key role in the regulation of mitogenic and metabolic activities. Therefore, structural characterizations of the molecular interactions involved in the IGF-I system are of large interest to further understand the structural biology of the system.

The solution structure of IGF-I has been determined from NMR spectroscopy data (Cooke et al., 1991). IGF-I shares the unique insulin-like fold with IGF-II and insulin (Terasawa et al., 1994). The IGF-I structure composes a central α-helix spanning residues 8−17 in the B-region between cysteine residues 6 and 18 and two smaller α-helical regions spanning amino acids 44−49 and 54−59, respectively, in the A-region of the molecule (Figures 1 and 2). Residues from these α-helical regions form the central hydrophobic core of the IGF-I folded structure, surrounded by structurally less well characterized regions spanning the C- and D-regions of the molecule (Cooke et al., 1991).

Previous investigations have identified the three amino-terminal residues of the B-region (Bagley et al., 1989) and further the whole B-helix (Oh et al., 1993b) and determinants in the A-region, amino acid residues 49, 50, and 51 (Clemmons et al., 1992), to be involved in IGFBP-1 binding. The IGF-I receptor binding epitopes have been mapped to determinants around amino acids 23−25 of the B-region, specifically to tyrosine 24, and to tyrosine 31 in the C-region, as well as the A-region tyrosine 60 (Cascieri et al., 1988; Bayne et al., 1990). These residues form parts of a hydrophobic patch located on the opposite side of the suggested IGFBP binding determinants (Figure 2). High-affinity IGF-I receptor binding determinants have further been identified to the C-region (Bayne et al., 1989) and more

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[1] Abbreviations: CD, circular dichroism; EDC, *N*-ethyl-*N*′-[(dimethylamino)propyl]carbodiimide; NHS, *N*-hydroxysuccinimide; IGF-I, insulin-like growth factor I; IGFBP-1, insulin-like growth factor binding protein 1; IGF-I^R, insulin-like growth factor I receptor; sIGF-I^R, soluble insulin-like growth factor I receptor extracellular portion; RU, resonance units.

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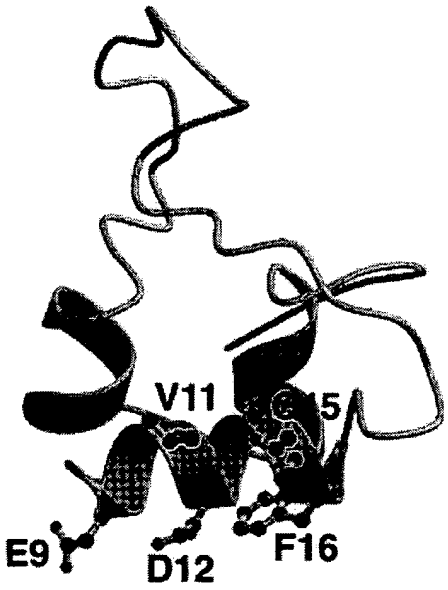

FIGURE 1: Ribbon representation of the three-dimensional structure of IGF-I adapted from the solution NMR structure (Cooke et al., 1991). The surface-exposed residues Glu 9(E9), Val 11(V11), Asp 12(D12), Gln 15(Q15), and Phe 16(F16), mutated in this study, are represented as ball-and-stick-type side chains and labeled; α-helices are drawn as ribbons. The representation was created using the software MOLSCRIPT (Kraulis, 1991) and RASTER 3D (Meritt & Murphy, 1994).

specifically to the charged residues Arg 36 and Arg 37 (Zhang et al., 1994).

In the present study, we have determined binding specificity contributions to both IGFBP-1 and soluble sIGF-I^R (sIGF-I^R) by the use of amino acid substituted IGF-I variants. Five single amino acid residue replacements in the B-region α-helix were produced: V11A, D12A, Q15A, Q15E, and F16A (Figure 2). The side chains of these residues are mainly exposed on the surface of the molecule, enabling possible involvement in intermolecular interactions. Since IGF-I possesses a thermodynamic inability to quantitatively form native disulfides *in vitro* (Miller et al., 1993; Hober et al., 1992), far-UV circular dichroism spectroscopy was utilized to detect possible changes in secondary structure and, when motivated, peptic digestion mapping to verify correct disulfide pairing. Surprisingly, it was found that most of the mutant IGF-I molecules showed decreased α-helicity resulting in reduced on-rate of binding to IGFBP-1 and sIGF-I^R. These results demonstrate the necessity to monitor structural changes in IGF-I upon amino acid substitutions, which is discussed.

EXPERIMENTAL PROCEDURES

DNA Preparations, Enzymes, and Bacterial Strains. DNA preparation and manipulations were performed using standard protocols essentially according to Sambrook et al. (1989). Restriction and modifying enzymes were from New England Biolabs or from Boehringer Mannheim (Mannheim, Germany). *Escherichia coli* RRI (Bolivar et al., 1977) was used for DNA manipulations and *E. coli* RV308 (Maurer et al., 1980) for protein production.

DNA Construction and in Vitro Mutagenesis. Amino acid substituted variants of IGF-I were constructed using a solid-phase *in vitro* mutagenesis protocol based on magnetic separation (Hultman et al., 1990). The method is based on the binding of biotinylated template DNA to streptavidin-coated magnetic beads (Dynabeads M280; Dynal AS, Oslo, Norway). The plasmid pRIT28:IGF-I was constructed by inserting a *Eco*RI−*Hin*dIII 210 base pair IGF-I gene fragment (Elmblad et al., 1984) into pRIT28 (Hultman et al., 1989), restricted with the same enzymes. The resulting plasmid was used to generate single-stranded DNA fragments used in the solid-phase mutagenesis. The vector fragment was restricted with *Eco*RI and biotinylated with Klenow DNA polymerase using biotin−dUTP (Boehringer Mannheim, Mannheim, Germany) and normal dATP, dCTP, and dGTP nucleotides at 0.5 mM each. The biotinylated vector was restricted with *Pvu*II and the resulting fragment bound to the magnetic beads via the streptavidin−biotin coupling. Washing the beads with 0.15 M NaOH released the minus strand of the vector fragment. The IGF-I insert was prepared in the same way by restriction with the *Bgl*II, biotinylated fill-in reaction followed by cleavage with *Eco*RV. The resulting bound fragment was made single stranded with alkali and a 26 base pair general primer 5′-GGC AGT GAG CGC AAC GCA ATT ATT GT- 3′ annealed. Depending on the mutation the following primers were phosphorylated and annealed: E9A, 5′-GTG CGG TGC TGC ACT GGT TGA CGC T-3′; V11A, 5′-GTG CTG AAC TGG CTG ACG CTC TGC A-3′; D12A, 5′-CTG AAC TGG TTG CCG CTC TGC AGT T-3′; Q15A, 5′-TTG ACG CTC TGG CTG TTG TTT GCG G-3′; F16A, 5′-GAC GTC CTG CAG GTC GTT TGC GGT G-3′. The sixth mutation, Q15E, was unexpectedly detected when sequencing a clone from the Q15A mutagenesis. The primers were extended and ligated in the same reaction by T4 DNA polymerase and T4 DNA ligase, respectively. Resulting mutated inserts were eluted from the solid phase with alkali and thereafter annealed to the prepared vector. The insert strand overlaps the vector strand, resulting in base pairing in the constant regions. The annealing mix was transformed directly into competent *E. coli* RR1 cells without extending the gapped duplex into double-stranded DNA.

DNA Sequencing. Nucleotide sequences were confirmed by DNA sequencing using dideoxy sequencing and dye terminators. The sequence was analyzed using an ABI 373A automated sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA).

Production Vector. The mutant IGF-I variants were subcloned into the production vector pKP 522 (Jansson et al., 1996) as an *Eco*RI and *Hin*dIII fragment. The vector used has a pUC-based origin of replication and kanamycin resistance as selectable marker. The transcription of IGF-I mRNA is initiated from the *E. coli trp* promoter.

Production and Purification of IGF-I Variants. IGF-I variants were produced intracellularly in *E. coli* as fusion proteins to a single IgG binding Z-domain derived from staphylococcal protein A (Nilsson et al., 1987). *E. coli* RV308 transformed with the production vector were grown at 30 °C overnight in 2 × YT medium supplemented with 60 mg/L kanamycin (Km). The overnight cultures were inoculated 1:50 in 1 × MJ minimal media (Jansson et al., 1996) containing 60 mg/L kanamycin and 4 g/L glucose and grown at 33 °C for 24 h. The protein production was induced at $OD_{600} = 0.5$ using 25 mg/L 3β-indoleacrylic acid (IAA). The cells were harvested by centrifugation at 4000*g* for 15 min. The cell pellet was resuspended in a solution of 6 M Gua-HCl, 150 mM NaCl, 50 mM KH_2PO_4, and 1 mM EDTA, pH 6.5. The cell slurry was further shaken overnight

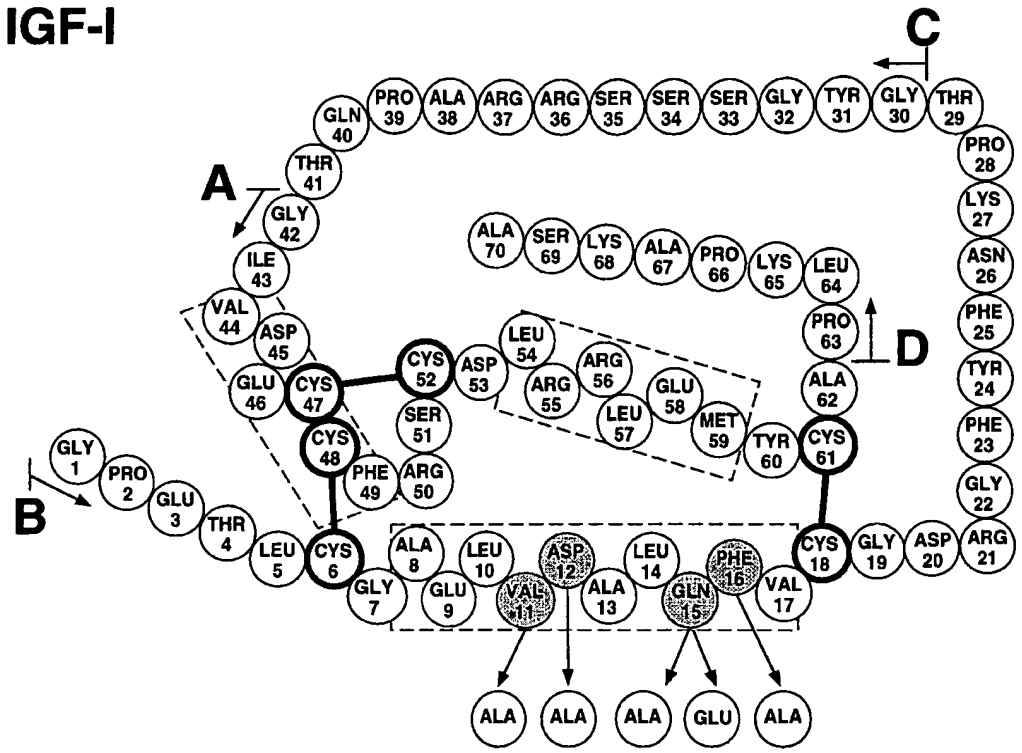

FIGURE 2: Primary structure of IGF-I with regions B, C, A, and D. The mutated residues in the B-region are shown in light gray. Helical regions are represented by broken-line boxes. The correct disulfide bond arrangement is represented by black bars.

at 4 °C. The initial purification by IgG affinity chromatography was performed by first diluting the resuspended cells to 1 M Gua-HCl concentration with 1 × TST (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20). After centrifugation at 15300*g* for 10 min, the supernatant was passed over an IgG−Sepharose FF column (Pharmacia Biotech, Uppsala, Sweden), equilibrated with 1 × TST. The column was washed with 10 column volumes of TST and 2 volumes of 5 mM ammonium acetate, pH 5.0. The bound fusion protein was eluted by 0.2 M acetic acid, pH 2.8. The protein eluate was subsequently lyophilized. Refolding and cleavage were performed by dissolving the lyophilized protein in a buffer containing 1.25 M Gua-HCl, 50 mM glycine, 1 mM EDTA, 20% ethanol, 0.125% Tween 20, and 10 μM reduced DTT at pH 9.0. Protein disulfides were formed by air oxidation at room temperature for 16 h. An equal volume of 2× cleavage mixture [4 M NaOH, 0.2 M Tris-HCl, 4 M hydroxyammonium chloride ($HONH_3Cl$)] was added, and the pH was adjusted to 9.5 using NaOH. The cleavage was performed at 45 °C for 5 h. The reaction was stopped by titrating to pH 6.6, using acetic acid, and desalting the sample on a Sephadex G25M column equilibrated with 150 mM ammonium acetate, pH 7.0. The fusion partner Z and remaining uncleaved fusion protein were removed by passing the desalted material over an IgG−Sepharose column equilibrated with 150 mM ammonium acetate, pH 7, using a 5 mL/min flow rate. The flow-through was collected and titrated to pH 4, using acetic acid.

The protein solution was rapidly frozen using a dry ice/ethanol bath and subsequently lyophilized before final purification. Remaining impurities and modified forms of IGF-I were removed by reverse-phase HPLC, using a Kromasil C8 column, 4.6 × 250 mm (Eka Nobel, Bohus, Sweden), on a HP 1090 HPLC system (Hewlett-Packard, Palo Alto, CA), equipped with diode array detection. The separating gradient used was 33−45% acetonitrile in 0.25% pentafluoropropionic acid (PFPA) over 50 min, at a flow rate of 1 mL/min and a temperature of 50 °C. The pure protein was lyophilized and stored in aliquots at −85 °C.

Native IGF-I was produced in analogy with the variants as a Z-fusion protein in *E. coli* purified as described (Forsberg et al., 1990; Moks et al., 1987).

Production and Purification of IGFBP-1. Recombinant IGFBP-1 was produced in DON cells transfected with a papilloma viral vector containing an expression cassette with the cloned human IGFBP-1 gene under the control of a BPV promoter (Luthman et al., 1989). IGFBP-1 was purified from conditioned cell medium by IGF-I affinity chromatography. After sample application, the column was washed with 5 column volumes of TST followed by 5 volumes of 5 mM ammonium acetate at a linear flow rate of 5 cm/min. Bound IGFBP-1 was eluted using 1 M acetic acid, pH 2.8, at the same flow rate. The eluted material was further applied to an S-Sepharose cation-exchange column (Pharmacia Biotech, Uppsala, Sweden), equilibrated with a buffer containing 90% 20 mM ammonium acetate, pH 4.5, and 10% 500 mM ammonium acetate, pH 7.0. After being washed with equilibration buffer, the IGFBP-1 was eluted with a linear gradient of 10−70% 500 mM ammonium acetate, pH 7.0, in 20 mM ammonium acetate, pH 4.5, over 30 min with a flow rate of 1 mL/min. The purified IGFBP-1 protein was finally lyophilized and stored at −20 °C.

Production of IGF-I Receptor. The soluble IGF-I receptor was produced as a secreted fusion to a synthetic IgG affinity-handle protein Z (to be described elsewhere). Briefly, the receptor fusion was secreted from a transfected 293 primary human kidney cell line, and the protein was recovered and purified from the growth media by applying an IgG affinity chromatography procedure.

Protein Analysis. Quantitative amino acid analysis was performed by acid hydrolysis followed by analysis using a Beckman 6300 amino acid analyzer, equipped with a System Gold data handling system (Beckman, Fullerton, CA). Protein homogeneity was evaluated by SDS−PAGE (Laemmli, 1970) or by RP HPLC.

Circular Dichroism. CD spectra were recorded using a Jasco J720 spectropolarimeter. Protein samples were dissolved in 10 mM potassium phosphate buffer, pH 7.0, to a final concentration of 0.1 mg/mL. Spectra were recorded from 250 to 184 nm at a step resolution of 0.1 nm and a scanning speed of 5 nm/min. Each spectrum is the average of five accumulated scans. Subsequently, actual concentration of each protein sample was determined by quantitative amino acid composition analysis. Secondary structure estimation was performed using the VARSLC1, variable selection secondary structure prediction software (Manavalan & Johnson, 1987) compiled for a Silicon Graphics Power Challenge server (Silicon Graphics, Mountain View, CA). The CD spectra of the IGF-I variants were compared to a CD library containing reference spectra of 33 proteins. Two proteins were excluded in each iteration, creating 528 possible combinations of the first fit. The two least comparable proteins were removed from the reference set, and the iterative fitting was repeated until the set structure content criteria, more than 96% total secondary structure classified and the RMS difference to the reference set <0.15, were satisfied.

Peptic Digestion. Peptic digestions were performed using a protein:enzyme ratio (w/w) of 10:1. A 30 μg portion of the protein to be digested was lyophilized. The protein was redissolved in 200 μl of 10 mM HCl containing 3 μg of porcine pepsin (Sigma, St. Louis, MO) and incubated at ambient temperature for 4 h. Peptic fragments were separated by reverse-phase HPLC on a Kromasil C8 column, 7 μm particles, 10 nm pore size (Eka Nobel, Bohus, Sweden), using a linear gradient from 0 to 45% acetonitrile and 0.1% TFA in 40 min at a flow rate of 1 mL/min and a column temperature of 30 °C. The eluting peaks were detected at 220 nm using a diode array detector on a Hewlett-Packard 1090 HPLC system.

Biosensor Analysis. The BIAcore, Sensorchip CM5 (certified grade), Surfactant P20, and amine coupling reagents, *N*-ethyl-*N'*-[(dimethylamino)propyl]carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine hydrochloride, were obtained from Pharmacia Biosensor (Uppsala, Sweden). All other buffer chemicals were obtained from Sigma or Fluka (Buchs, Switzerland). All kinetic measurements were performed with the larger molecule IGFBP-1 or sIGF-I^R as the immobilized acceptor molecule. Immobilization was performed at 5 μL/min in 1 × HBS (10 mM HEPES, pH 7.4,150 mM NaCl, 3.4 mM EDTA, 0.05% P20) as the driving buffer. IGFBP-1 was dissolved in 50 mM sodium acetate, pH 4.7, at a concentration of 50 μg/mL and was immobilized via primary amine groups as previously described (Löfås & Johnsson, 1990), utilizing EDC/NHS carbodiimide coupling reagents, to a final resonance value between 700 and 2000 RU. The IGF-I receptor immobilization was performed by a similar procedure at pH 4. Final levels of sIGF-I^R immobilization were 6500−7500 RU. All kinetic experiments were performed using 1 × HBS as the driving buffer at a flow rate of 8 μL/min. The injection of analyte was controlled using the *kinject* command in the Bialogue control software. Each sample was injected twice at five different concentrations in random order over the same surface in each measurement series. The concentrations used in IGFBP-1 kinetics were, for native IGF-I and all mutants (except for F16A), 192, 96, 48, 24, and 12 nM, respectively, and, for F16A, 768, 384, 192, 96, and 24 nM. Each protein was analyzed using at least three separate experiments, using independent acceptor molecule immobilizations. Kinetic measurements were performed by injection of each analyte for 300 s followed by dissociation in buffer flow for 400 s. The immobilized IGFBP-1 was regenerated after each cycle using a 12 μL injection of 100 mM HCl. IGF-I ligand concentrations used in receptor measurements were, for native IGF-I and mutants, except F16A, 524, 262, 131, 66, 33, and 16.5 nM and, for F16A, 2096, 1048, 524, 262, 131, and 66 nM. The immobilized sIGF-I^R surface was regenerated after each cycle by injection of 12 μL of acidic regeneration solution containing 0.3 M sodium citrate, pH 5, and 0.4 M NaCl. Ligand samples were injected twice at six different concentrations in random order over the same surface in each measurement series. The temperature in all kinetic experiments was 298 K.

BIAcore Evaluation. Kinetic parameters were calculated using the kinetics evaluation software package BIAevaluation 2 (Pharmacia Biosensor, Uppsala, Sweden). Theories behind BIAcore measurements, evaluations, and calculations have been extensively described previously; for a review see Karlsson et al. (1994).

Briefly, k_{on} was calculated using the equation:

$$dR/dt = \text{constant} - [[(k_{on}C) + k_{off}]R] \quad (1)$$

The dR/dt is the relative change in resonance signal per time unit, C is the concentration of ligand in the flow, and R is the relative response. An approximate k_{off} could be obtained in the analysis of the association phase (see eq 1 above).

However, this value can more accurately be determined by kinetic measurements of ligand dissociation in a buffer flow. From dissociation data, k_{off} can be calculated using the equation:

$$\ln(R1/Rn) = k_{off}(tn - tl) \quad (2)$$

The parameter Rl is the relative response at time tl, and Rn is the relative response at time tn. The affinity constant, K_{aff}, was calculated from the kinetic constants using the equation:

$$K_{aff} = k_{on}/k_{off} \quad (3)$$

Differences in free energy of binding , $\Delta\Delta G$, between each mutant protein, respectively, and wild type were calculated from the affinity constants using the equation:

$$\Delta\Delta G = -RT \ln[K_{aff}(\text{mutant})/K_{aff}(\text{wt})] \quad (4)$$

Ligand Binding Assay. Ligand binding assays were performed in duplicates using 100 mM sodium phosphate, pH 7.5, 150 mM NaCl, and 0.05% Tween 20, as assay buffer and ^{125}I-labeled IGF-I (Amersham, Bucks, U.K.). Pre-IgG-coated Scintistrip microwell plates (Wallac, Turku, Finland) were incubated with the receptor preparation. Plates were washed three times with PBS-T and three times with assay buffer. One series of dilutions was incubated with ^{125}I-IGF-I (20 000−30 000 cpm/0.1 mL). The second series was incubated with ^{125}I-IGF-I and unlabeled recombinant IGF-I

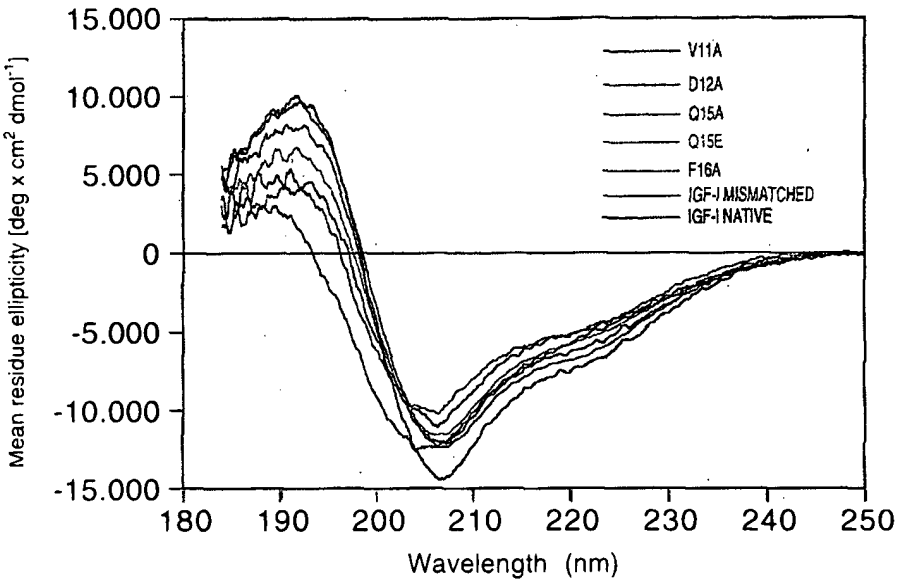

FIGURE 3: Superposition of the far-UV CD spectra of native IGF-I and mutated variants from 184 to 250 nm.

(1 μM) for control of unspecific binding. After incubation at 4 °C for 3 h or at room temperature for 1 h, plates were rapidly washed with assay buffer and allowed to dry. For competitive ligand binding assays, plates were coated with a defined receptor concentration and washed. Receptors were incubated with 100 μL of ^{125}I-IGF-I in all wells. Secondly, serial dilutions were made starting from 50 μL of ^{125}I-IGF-I solution containing 3 μM unlabeled IGF-I. After incubation, wells were treated as described above. Retained radioactivity was measured using a γ counter (Wallac, Turku, Finland).

Electrostatic Potential Calculations. Isoelectric contour surface plots of IGF-I and mutants were created using the Delphi module (Gilson & Honig, 1988a,b; Gilson et al., 1987) in the Insight II software package (Molecular Simulations Inc., San Diego, CA) on a Silicon Graphics interface. Potentials were calculated using the PDB coordinates of the NMR solution structure, PDB entry 2gf1 (Cooke et al., 1991). The charge of the molecule was calculated at pH 7.4 using the cvff force field. The boundary was set to full Colombic with a border of 10 with 65 maximum grid points. The solute dielectric was set to 4 using current charges, and the solvent dielectric was 80 with a radius of 1.4.

RESULTS

The intention of this work has been to determine the contribution of single amino acid residues of IGF-I on its binding specificity to soluble IGF-I^R and IGFBP-1, respectively. It was decided to create a defined experimental system where all molecular components are highly purified recombinant material and where one interaction at a time is studied *in vitro*, without other influencing factors such as additional interactions possibly found in cellular receptor assays. In addition, since IGF-I is known to possess a thermodynamic folding inability to quantitatively reach its native form (Hober et al., 1992), it was decided to carefully analyze disulfides and secondary structures of produced IGF-I variants. The amino acid substituted variants of IGF-I were originally designed as an alanine scan of the mostly surface exposed residues of the N-terminal α-helix, residues 9, 11, 12, 15, and 16. These residues were selected from analysis of surface-exposed area, using the Protable module contained in Sybyl 6.22 (Tripos, St. Louis, MO) and the NMR-based IGF-I structure PDB entry 2gf1 (Cooke et al., 1991). The genes encoding the single amino acid substituted IGF-I proteins were successfully constructed at the DNA level using a solid-phase mutagenesis protocol (Hultman et al., 1990). The Q15E variant was unexpectedly detected when sequencing Q15A clones. The different IGF-I variants were produced as fusions to the IgG binding Z-domain (Nilsson et al., 1987). The advantages of using the Z-fusion protein approach to overproduce IGF-I are well established (Nilsson et al., 1990). The Z-fusion approach, in addition to providing a uniform initial affinity purification protocol for mutated proteins with altered biochemical properties, also enhances production levels, solubility, and stability against degradations during expression and purification (Nilsson & Abrahmsén, 1990). In addition, refolding yields are typically significantly improved compared to the unfused product protein (Samuelsson et al., 1991).

The amino acid substituted variants of IGF-I [IGF-I(V11A), IGF-I(D12A), IGF-I(Q15A), IGF-I(Q15E), IGF-I(F16A)] were produced in *E. coli* and purified to homogeneity. The amino acid composition contents were according to what was expected from the respective deduced amino acid sequences (data not shown). The purity of the produced variants was more than 95% as determined by RP HPLC (data not shown). The gene-constructed IGF-I(E9A) variant was not successfully produced as full-length material despite several attempts. No correctly folded full-length material could be recovered after refolding, and further attempts to purify this variant were not undertaken. IGFBP-1 and sIGF-I^R were produced and purified to homogeneity. The purity of these produced recombinant proteins was confirmed by SDS−PAGE and estimated to more than 90% (data not shown).

Structural Characterization of IGF-I Variants. The far-UV CD spectra for all purified IGF-I variants display deviations compared to native IGF-I (Figure 3). The spectra shown are normalized to the same concentration using quantitative amino acid analysis of the samples after CD measurements.

Table 1: Listing of the Fraction of Secondary Structure from Variable Selection Analysis of Circular Dichroism Spectra of IGF-I and IGF-I Variants

structure[a]	IGF-I	mmIGF-I	IGF-I(V11A)	IGF-I(D12A)	IGF-I(Q15A)	IGF-I(Q15E)	IGF-I(F16A)
H	0.22	0.13	0.15	0.20	0.18	0.15	0.13
A	0.15	0.23	0.25	0.21	0.29	0.23	0.18
P	0.06	0.06	0.06	0.05	0.03	0.07	0.05
T	0.26	0.25	0.22	0.25	0.25	0.24	0.27
O	0.30	0.32	0.33	0.29	0.25	0.31	0.37
total	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[a] H, α-helix; A, antiparallel β-sheet; P, parallel β-sheet; T, β-turn; O, other structure.

Spectra of IGF-I(D12A), IGF-I(Q15A), and IGF-I(Q15E) are mostly similar but not identical to the spectrum of that of native IGF-I. The spectra for IGF-I(V11A) and IGF-I(F16A) exhibit some larger deviations compared to that of native IGF-I. Native IGF-I and V11A have identical spectra in the 250−230 nm region whereas mismatched IGF-I and V11A have similar spectra at 210−184 nm where the amplitudes at lower wavelengths are lower than that of the native spectra. The IGF-I(F16A) spectrum displays the largest differences from the native one. The two spectra are similar in the 250−210 nm region, but F16A has drastically lowered amplitudes in the region 205−184 nm. The spectra from V11A or F16A are different from those of both native IGF-I and mismatched IGF-I. Common to all variants is a decrease in amplitude compared to native IGF-I. The change in secondary structure content of the variants was analyzed using the variable selection method as described. All IGF-I variants fulfilled the set criteria of 96−100% total secondary structure in the analysis. The results are summarized in Table 1. The largest difference in structure content between the variants is the fraction of α-helical content. The mutants with the largest deviations, V11A and F16A, come close to the mismatched IGF-I structure content.

The variants showing the largest aberrations in CD spectra compared to native IGF-I, V11A and F16A, were digested with pepsin and the fragments separated by RP HPLC in order to determine the disulfide bond pattern. Separation of IGF-I pepsin fragments exhibits different characteristic patterns dependent on the disulfide pairing of the molecule. This method thus allows the distinction between native and mismatched disulfide pairing in IGF-I. The elution time of the major peptic fragment of IGF-I is approximately 18 min in the chromatogram (Figure 4A). This peak, labeled 1, corresponds to the disulfide-linked fragments 16−24 and 60−70 of IGF-I (Forsberg et al., 1990). The elution profile for the V11A peptide fragments (Figure 4C) is essentially identical to that of native for all peaks. The V11A variant is therefore concluded to have the correct disulfide pairing pattern. The data for F16A (Figure 4D) are less conclusive, and the cleavage pattern does not fully correlate to native nor mismatched patterns (Figure 4B). For F16A the major peak, 1*, elutes at approximately 20 min (Figure 4D). This is most likely the result of the removal of the major cleavage site between residues 15 and 16. This should result in the larger fragments 14−24, 60−70 and 11−24, 60−70 having potentially altered elution times. The different cleavage pattern from F16A is therefore concluded to arise from altered cleavage sites rather than mismatched disulfide variants.

Biosensor Analysis. BIAcore real time biosensor kinetic measurements were performed using either IGFBP-1 or sIGF-I^R as the immobilized acceptor molecule. Sensorgrams for the interaction of IGF-I and variants to IGFBP-1 are

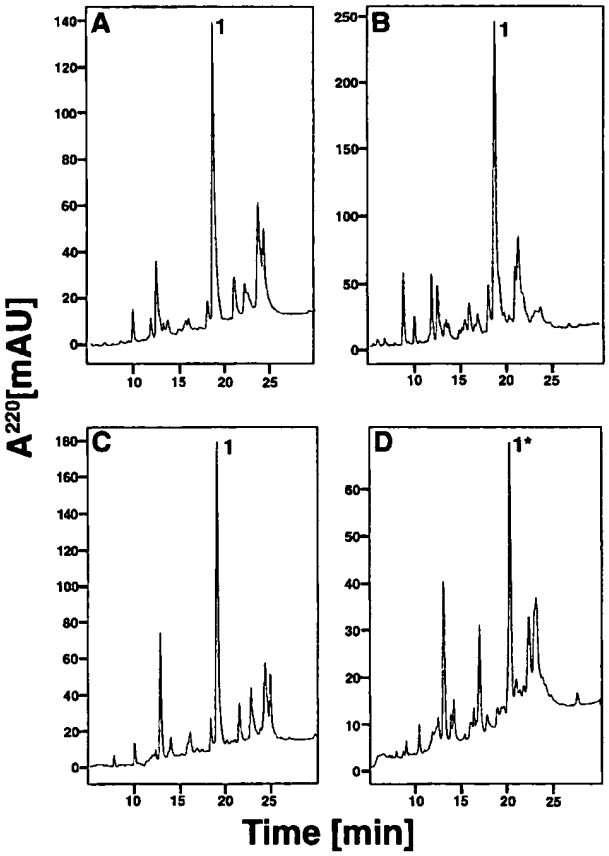

FIGURE 4: Chromatograms of reverse-phase HPLC analysis of peptic-digested IGF-I variants. The absorbance was monitored at 220 nm. A: Native IGF-I cleavage pattern. B: Incorrectly disulfide-paired IGF-I (mismatch). C: Cleavage pattern of the variant V11A. D: Cleavage pattern of the variant F16A.

shown in Figure 5A,B and interactions to sIGF-I^R in Figure 5C,D. A summary of the calculated rate and equilibrium constants is found in Table 2. IGF-I affinity was determined in three separate runs using duplicate injections of each concentration. The cumulative error in the determined association constants from all runs was estimated to be less than 12%, when calculated as the square root of the sum of the squares of the errors in amino acid analysis, pipetting, and data fitting. The binding to IGFBP-1 is reduced for all the mutants except D12A, which has a 2.7-fold increase in measured association constant. The binding to sIGF-I^R is reduced for all of the mutants.

Radiolabeled Receptor Assay. Competitive radiolabeled receptor assay was performed as a control experiment to verify the affinities measured by biosensor technology. Purified receptor was immobilized through the IgG(Fc)−Z-protein interaction on IgG-coated plastic plates. The measured relative affinities of the IGF-I variants score in the

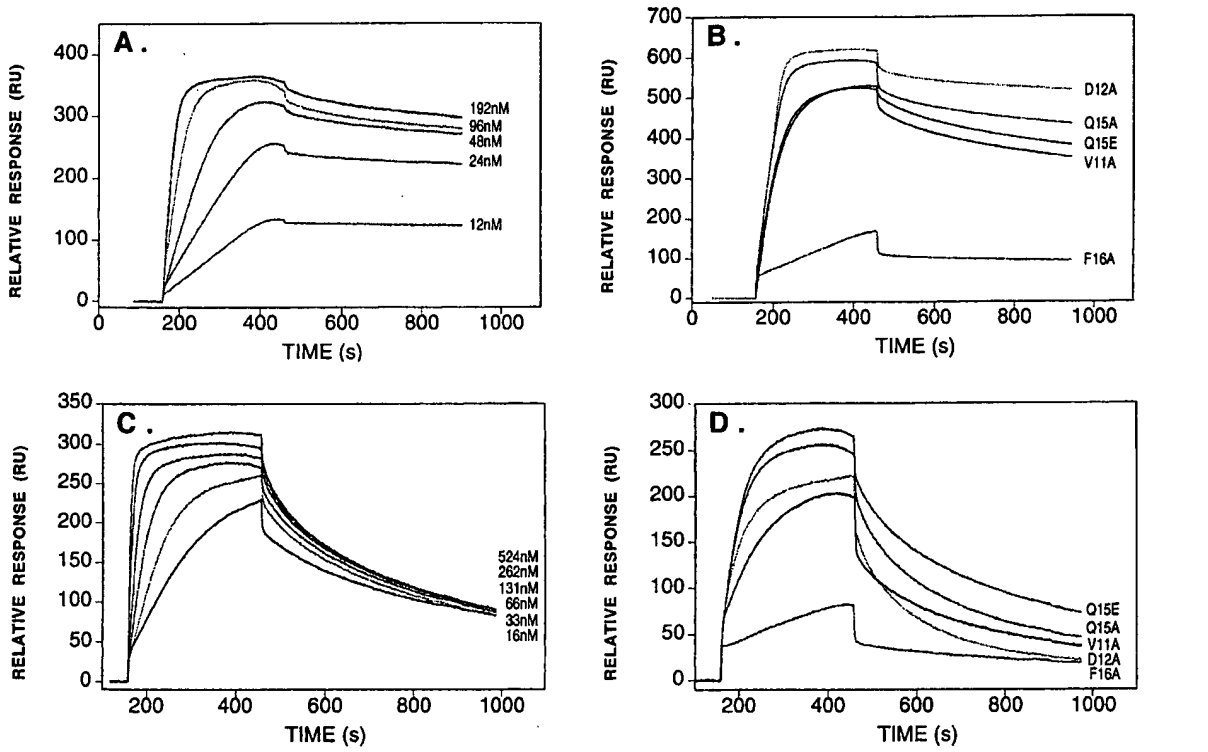

FIGURE 5: Biosensor analysis of ligand binding to immobilized IGFBP1 and sIGF-I^R. The relative response in resonance units (RU) plotted against time. A: Sensorgrams showing the interaction of IGF-I to IGFBP1 at the ligand concentrations indicated in the graph. B: Representative sensorgram of the different IGF-I variants at 96 nM concentration interacting with immobilized IGFBP1. C: Sensorgrams showing the interaction of IGF-I to sIGF-I^R at the indicated ligand concentrations. D: Representative sensorgram of the different IGF-I variants at 66 nM concentration interacting with immobilized sIGF-I^R.

Table 2: Kinetic Parameters from Biosensor Analysis of the Interaction between IGF-I and Variants Thereof Interacting with IGFBP-1 and sIGF-I^R, Respectively

		IGF-I	V11A	D12A	Q15A	Q15E	F16A
k_{on} (M^{-1} s^{-1} × 10^{-5})	IGFBP-1	6.12	2.55	8.61	4.91	2.79	0.153
	IGF-I-REC	4.65	2.62	2.40	3.43	3.77	0.149
k_{off} (s^{-1} × 10^4)	IGFBP-1	3.47	5.62	1.79	4.09	4.93	4.36
	IGF-I-REC	16.7	23.2	32.0	24.4	19.7	19.9
K_A (M^{-1} × 10^{-9})	IGFBP-1	1.76	0.45	4.81	1.20	0.57	0.035
	IGF-I-REC	0.278	0.113	0.075	0.141	0.191	0.0075
$\Delta\Delta G$ (kcal/mol)	IGFBP-1	[0]	0.80	−0.59	0.23	0.67	2.32
	IGF-I-REC	[0]	0.53	0.78	0.40	0.22	2.14

same order as in the BIAcore assay (Figure 6).

DISCUSSION

In this work, interactions between human IGF-I and its acceptor molecules IGFBP-1 and sIGF-I^R have been investigated by use of produced and extensively characterized single amino acid substituted variants of IGF-I and biospecific interaction analysis methodology (BIAcore). The initial aim was to investigate the effect of single amino acid substitution on the different IGF-I interactions to further establish important structural determinants of the molecule. The mutants were effectively produced and refolded as fusion proteins and purified to homogeneity using an affinity handle fusion protein approach. The Z-fusion protein approach facilitates the purification by providing a uniform initial purification step for mutant proteins displaying differences in both isoelectric point and hydrophobic properties. Further purification after specific removal of the affinity handle included high-resolution RP HPLC where also mismatched forms are removed.

Most of the substitutions in the B-region α−helix decrease the IGF-I binding affinity to both sIGF-I^R and IGFBP-1. However, as will be further outlined in the following, these altered affinities are concluded to result not only in the removal of specific interactions through amino acid substitution but also in changing the global structure of IGF-I. In the first mechanism, the residue is concluded to be directly involved in an interaction resulting in a faster off-rate of binding. In the second mechanism, the residue is affecting the fold as seen in a slower on-rate of binding. We believe that any smaller or larger substitution of the structural determinants in the highly ordered A- and B-regions of IGF-I may induce changes in three-dimensional packing of the molecule and hence influence binding affinity.

A clear correlation between on-rate and protein α-helicity is observed in the IGFBP-1 binding of the different IGF-I mutant proteins (Figure 7). A decrease in α-helicity is accompanied by a reduced on-rate to IGFBP-1 in an almost linear fashion. In contrast, the off-rates are not as influenced by the amino acid substitutions as are the on-rates (Table 2). This observation is in contrast to what was found for a large number of human growth hormone (hGH) mutant proteins and their binding to hGH binding protein (hGHBP) (Cunningham & Wells, 1993). In this study, almost all

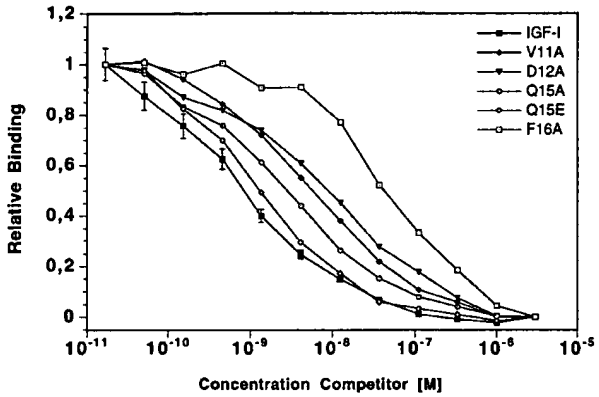

FIGURE 6: Competitive radiolabeled receptor assay performed using purified and immobilized sIGF-I^R. The half inhibitory concentration, IC_{50}, of the different IGF-I variants is estimated to M × 10^9: IGF-I, 2.0; V11A, 0.45; D12A, 0.20; Q15A, 0.8; Q15E, 0.95; F16A, 0.07. The displacement curves are the average of two separate series of duplicate measurements. As an indication of assay variability, standard errors of the mean are indicated for the IGF-I data when they are sufficiently large in relation to the symbols. The relative binding is normalized to 1.0 at the lowest concentration of competitor and to 0.0 at the highest concentration for an easier comparison of the different ligands.

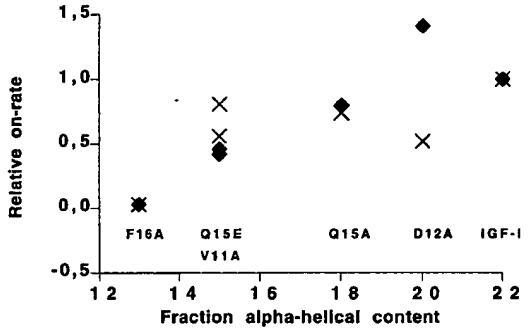

FIGURE 7: Plot of the relative on-rate as a function of the α-helical content of the IGF-I variants for the interaction to IGFBP-1 (◆), and to sIGF-I^R (×). The linear regression correlation for the relation between α-helicity and *decreased* IGFBP1 on-rate is 0.95.

mutant hGH molecules with reduced hGHBP binding showed a faster off-rate of binding. The IGF-I mutant with the largest shift in structure, F16A, has only a 25% increase in off-rate compared to native IGF-I, whereas the V11A mutant with a slightly less altered structural content has an increased off-rate by 62%. This could be interpreted as an induced fit of IGF-I(F16A) upon IGFBP-1 association and that V11 is more involved in stabilizing the IGFBP-1 interaction. Thus F16 is probably more contributing in stabilizing the IGF-I structure, in spite of the fact that it has been concluded from NMR spectroscopy constraints to be mainly a surface-exposed residue (Cooke et al., 1991).

The potentiating effect of D12A on IGFBP-1 association rate might have several explanations. Analysis of the electrostatic surfaces using Delphi was performed on IGF-I, IGF-II, insulin, and the described mutants. The results of the analysis show that IGF-I is a electrostatically polarized protein with a negatively charged N-terminus and the first part of the B-region helix as one continuous negatively charged patch (data not shown). Insulin and IGF-II do not show the same highly polarized charge pattern as IGF-I, which may contribute to the different binding specificities of the different IGF molecules. The C-region and the proposed receptor interaction site display a continuous positively charged region. A zero net charge plane cut through the molecule at the N-terminal end of the N-terminal helix dividing the molecule into two halves that overlap rather well to the proposed IGF-I^R binding side on one side and to the proposed IGFBP binding side on the other. The net zero plane coincides with the previously identified receptor-specific residues 23, 24, 25, and 31, and the IGFBP binding determinants are mostly localized around the N-terminal negatively charged region. The D12A mutant makes a hole in the continuous charge distribution along the N-terminal α-helix and expands the uncharged region at the C-terminal end of the helix. The change in charge and hydrophobicity at this position seems to be important in the contribution to the increase in IGFBP-1 binding and the decrease in IGF-I^R binding. This might be the case if D12 is actually hindering IGFBP-1 interaction but is necessary for receptor binding. Since V11A has larger effect on IGFBP-1 binding than receptor binding, we suggest position 12 to be at the boundary of receptor/BP binding specificity determinants. This assumption is supported by the fact that the observed change in affinity of D12A, influencing both receptor and IGFBP1 binding, seems to be mostly a change in side chain specificity rather than a structurally affected mutant. The observed reduction of α-helicity in the Q15E mutant may be attributed to the introduction of a negatively charged amino acid close to D12; the two residues are *i*, *i* + 3 neighbors on the helix. The structural proximity may give rise to electrostatic repulsion resulting in changes in the structure. The α-helical content of Q15A is higher than that for Q15E, indicating that the change from an uncharged residue to another one makes less distortions of the structure, compared to introduction of a charged side chain. Q15A scores as second best binder to IGFBP-1 and binds better than Q15E; the reverse is seen for sIGF-I^R binding. The introduction of the negative charge in this position thus seems to influence IGFBP-1 binding to a larger extent than sIGF-I^R binding.

The affinity for sIGF-I^R is decreased for all analyzed mutant IGF-I proteins including D12A. The D12A variant shows both reduced on-rate and an almost twice as fast off-rate compared to native IGF-I. These drastic effects strongly suggest amino acid 12 to be directly involved in receptor binding. However, measured decreases in on-rates for receptor binding do not correlate as well with reduced α-helicity as was found in the interaction with IGFBP-1 (Figure 7), implying that other involved determinants are contributing in the sIGF-I^R interaction. Also in the sIGF-I^R interaction, the off-rate is close to the native rate for F16A, expanding the induced fit model to the receptor interaction and suggesting again that this residue is mostly stabilizing IGF-I structure rather than contributing to direct involvement in binding. Interestingly, we recently demonstrated by titration calorimetric measurements that the binding of IGF-I to sIGF-IR is accomplished by a rather large decrease in entropy [$\Delta S = -70$ cal/(K mol) at 37 °C] and decrease in heat capacity ($\Delta C_p^o = -0.5$ cal/(K mol)], indicative of a structural change of the system (to be published elsewhere). Thus, the structural changes that we observe in the mutant proteins may reflect an intrinsic property of the IGF-I ligand that is normally part of the receptor binding mechanism.

Far-UV CD analysis demonstrates that all of the analyzed mutant IGF-I molecules display different spectra compared with the spectrum of native IGF-I. The least affected spectra

are those for the D12A, Q15A, and Q15E variants. Variable selection secondary structure analysis of the CD spectra suggests only minor decrease in α-helicity for these three IGF variants. These mutants were assumed to be essentially correctly folded on the basis of the CD spectra and RP HPLC analysis. However, IGF-I(V11A) and IGF-I(F16A) variants show larger deviations in their CD spectra, and their spectra resemble a closer similarity with the mismatched IGF-I. Therefore, these mutants were further analyzed by peptic digestion and separation of the cleaved fragments by RP HPLC. Both the correctly disulfide-paired protein and the mismatched give rise to specific separation patterns (Figure 4A,B). A thorough characterization of the IGF-I peptide cleavage fragments has been described earlier (Forsberg et al., 1990). The IGF-I(V11A) variant was found to display a separation pattern similar to that of native IGF-I (Figure 4C). Thus, purified IGF-I(V11A) is concluded to possess the disulfide bond pairing corresponding to native IGF-I, and the significant aberration of its CD spectrum must therefore have other explanations. The pepsin digestion pattern of IGF-I(F16A) does not match any of the other cleavage patterns (Figure 4D). The main reason for this result is most likely the removal of the dominant pepsin cleavage site in IGF-I, at the N-terminal side of F16. Further, changes in hydrophobicity of the different fragments due to removal of a phenyl group may alter elution properties of the fragments.

Variable selection analysis of CD data does not give further conclusive evidence that the disulfide pairing of purified IGF-I(F16A) is correct; mismatched IGF-1 is calculated to have 13% α-helical content and native 22%. The V11A mutant has 15% α-helix content and F16A 13%. Since V11A has correct disulfide pairing based on the peptide mapping, the reason for the reduced α-helical content may be distortion of the native core of hydrophobic packing. The side chain of V11 is surface exposed to 68%; the removal of the γ-methyl groups of V11A by introduction of the smaller unbranched alanine side chain is likely to affect the packing of the molecule and thus the secondary structure content. Furthermore, the net negative ellipticity contribution is sensitive to the context of the amino acids; this may further explain the differences between the IGF-I(V11A) spectra and the native IGF-I. The large structural changes observed in the F16A mutant, in the same order of α-helical reduction as in mismatched IGF-I, might be accounted for by the position of the aromatic side chain in the native structure. In the NMR structure (Cooke et al., 1991), the aromatic side chain is solvent exposed to as much as 53% (Figure 1), but the F16 side chain also covers parts of the core between the N-terminal (helix 1) and the C-terminal helix (helix 3). We conclude that the aromatic side chain of residue 16 is involved in stabilizing the IGF-I structure rather than being involved directly in any of the specific protein−protein interactions analyzed in this paper. The critical role of F16 in maintaining the structural integrity of IGF-I is an important finding in this paper.

The relatively high percentage of β-structure found in the variable selection analysis, but not in the solution structure, indicates only that CD and variable selection analysis are aimed more to fingerprint a specific structure than to quantitate the secondary structures in absolute terms. The C-region is further mostly unstructured in the reported solution structures; the CD spectra might contain contributions from this region appearing more as β-structure than random structure.

IGF-I has a very rapid acid proton exchange rate; all but about 10 of the interchangeable protons are exchanged within 3 min at neutral pH as measured by H/D exchange and analysis by mass spectrometry (P. Persson and B. Norén, personal communication). In the solution structure of IGF-II (Torres et al., 1995), slow proton exchange was limited to almost only hydrogen bonds within the stable α-helical regions. All of the insulin-like growth factors have a fairly small number of residues involved in the formation of the hydrophobic core, only 8−10 residues out of 70. Therefore, the IGF-I structure is assumed to be less rigid than a majority of proteins with a larger or tighter packed hydrophobic core. These findings highlight the necessity to perform structural analysis to explain contributions of the various IGF-I interactions. As demonstrated here, reduced α-helicity in IGF-I may have other explanations besides incorrect disulfide pairing, as for the V11A variant. Even though the F16A disulfide pattern was not mapped conclusively, we propose that the cysteine pairing is correct. A strong support for this conclusion is the fact that F16A has detectable affinity for the IGF type I receptor and mismatched IGF-I has not been found to possess any detectable receptor binding using a sensitive displacement assay (Forsberg et al., 1990). If the purified F16A variant was mismatched, it is unlikely that it would possess higher receptor affinity than the mismatched form with the native amino acid sequence.

The formation of native disulfide bonds in IGF-I is not quantitative *in vitro*, as demonstrated earlier (Miller et al., 1993; Raschdorf et al., 1988; Forsberg et al., 1990; Hober et al., 1992). The presence of two separate three-disulfide-bonded forms of the mature protein, the native form (disulfides 6−48, 47−52, and 18−61) and the mismatched form (disulfides 6−47, 48−52, and 18−61), motivates detailed structural analysis of purified mutated proteins in order to correctly interpret binding kinetic data and structure−function relationships. The basis for this suggestion is the difference in secondary structure content between the two folding variants and the reduced biological activity of the mismatched form (Forsberg et al., 1990). The production of IGF-I and variants thereof must consequently take into account the possible occurrence of mismatched species, and specific purification of each variant, with the disulfide bond pattern of the native form, must be performed. These special IGF-I properties have been neglected in previous IGF-I mutagenesis studies. Analysis of secondary structure from CD data has proven to discriminate subtle differences in secondary structure content in the IGF-I molecule. The mismatched form is easily identified, and mutants lacking either the 6−48 or 47−52 disulfide bonds display significant changes in far-UV CD spectra (S. Hober and B. Nilsson, submitted for publication).

When the results of this study are compared to previous examples of mutational analysis, performed using single amino acid substituted variants and BIAcore technology, a different influence is seen on binding kinetics. In the study of the hGH−hGHBP interaction (Cunningham & Wells, 1993), most of the effects of amino acid substitution were seen as an increased off-rate of binding. Further, in the hGH study, single amino acid substitutions resulted in either an increased off-rate or a decreased on-rate, but not by affecting both. In the works on the Z-domain of protein A (Cedergren et al., 1993, Jendeberg et al., 1995), mutants were found that influenced either the on- or off-rate or both. The hGH and Z studies share the common feature that the protein backbone

structure is concluded to be intact. In contrast, the IGF-I structure is found to be sensitive to structural disturbance caused by substitutions of surface-exposed amino acid residues.

In this paper we have concluded that the structure of IGF-I is very sensitive to amino acid substitutions. This rather extreme feature of the IGF-I molecule, the thermodynamic folding problem (Miller et al., 1993; Hober et al., 1992), the polarized charge distribution, and the small hydrophobic core (Cooke et al., 1991) all contribute to make mutational analyses less straightforward than what is normally the case. The presented data strongly suggest that mutational analysis of IGF-I must take into account both functional and structural aspects to enable interpretation of the binding data. The use of binding kinetic analyses combined with CD spectroscopy is one recommended approach to be able to separate changes in structures from direct involvement in binding.

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The Roles Of Tyrosines 24, 31, and 60 in the High Affinity Binding of Insulin-like Growth Factor-I to the Type 1 Insulin-like Growth Factor Receptor*

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A series of insulin-like growth factor I (IGF-I) structural analogs in which one or more of the three tyrosine residues were replaced with nonaromatic residues were produced and their binding properties characterized. The single point mutations, [Leu^{24}]IGF-I, [Ala^{31}]IGF-I, and [Leu^{60}]IGF-I result in an 18-, 6-, or 20-fold loss in affinity, respectively, for the type 1 IGF receptor. Multiple mutations, [Ala^{31},Leu^{60}]IGF-I, [Leu^{24}, Ala^{31}]IGF-I, [Leu^{24},Leu^{60}]IGF-I, or [Leu^{24},Ala^{31}, Leu^{60}] IGF-I result in a 520-, 240-, 1200-, or >1200-fold loss in affinity, respectively, at the type 1 IGF receptor. In contrast, none of the analogs display greater than a 2-fold loss in affinity for the acid-stable human serum binding proteins. At the insulin receptor, [Ala^{31}]IGF-I and [Leu^{24}]IGF-I are equipotent to and 5-fold less potent than IGF-I, whereas [Leu^{60}]IGF-I and the multiple mutation analogs are inactive up to 10 μM. Analogs [Leu^{24}]IGF-I, [Ala^{31}]IGF-I, and [Leu^{24},Ala^{31}]IGF-I are equipotent to IGF-I at the type 2 IGF receptor, whereas all analogs containing Leu^{60} demonstrate little measurable affinity at this receptor. Thus, Tyr^{24}, Tyr^{31}, and Tyr^{60} are involved in the high affinity binding of IGF-I to the type 1 IGF receptor, while Tyr^{60} is important for maintaining binding to the type 2 IGF receptor.

Human insulin-like growth factor I (hIGF-I)[1] is a 70-amino-acid protein purified from human serum (1) that promotes cell growth and differentiation of various cell types (2). The biological effects of hIGF-I are mediated through its binding to one or more of three receptors. hIGF-I binds with highest affinity to the type 1 IGF receptor, a receptor structurally homologous to the insulin receptor (3, 4). hIGF-I also cross-reacts with lower affinity to the insulin receptor and with the type 2 IGF receptor (3). hIGF-I binds with high affinity to specific soluble binding proteins (5, 6). These proteins may modulate the biological activities of hIGF-I (7–9).

Our laboratories have been mapping binding determinants on hIGF-I for its receptors and binding proteins by analyzing the binding properties of specific IGF-I analogs produced by recombinant DNA expression in yeast (10–13). These studies indicate that Tyr^{24} in the B region of hIGF-I is involved in binding to the type 1 IGF receptor (10). Deletion of the C region of IGF-I also reduces the affinity of IGF-I to the type 1 receptor (13). The position of Tyr^{31} in this C region suggests that this residue may be involved in type 1 receptor binding. The tyrosine at position 60 of hIGF-I is analogous to Tyr^{A19} of insulin. This residue in insulin is required to maintain binding of insulin to its receptor (14). Studies by Maly and Luthi (15) demonstrate that all three tyrosines of hIGF-I are protected from iodination when bound to the type 1 IGF receptor. To examine directly the roles of Tyr^{24}, Tyr^{31}, and Tyr^{60} in the binding properties of hIGF-I we generated single, double, and triple point mutations in these residues and determined the binding characteristics of the analogs.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, and T4 DNA ligase were from New England Biolabs and used according to their recommendations. Crystalline bovine serum albumin came from Sigma. Bio-Rex 70 and Bio-Gel P10 were from Bio-Rad. [^{125}I]Iodine was from Amersham and purified multiplication stimulating activity (rat IGF-II) was a gift from Dr. James Florini, Syracuse University.

DNA Manipulations—Standard DNA manipulations were carried out as described elsewhere (16). Oligodeoxynucleotides were synthesized by phosporamidite chemistry on an Applied Biosystems Model 380A synthesizer and gel purified as previously described (17). DNA sequences were determined on M13 templates or plasmid templates by the dideoxynucleotide chain termination method (18, 19). Plasmid strains were propagated in *Escherichia coli* strain DH5 following standard transformation procedures (20). Two rounds of transformations were carried out to avoid possible sequence heterogeneity in plasmids constructed with synthetic oligodeoxynucleotides.

Design and Construction of Mutant hIGF-I Genes—The construction of [Tyr^{24}]IGF-I was described previously (10). Plasmid pJY31 encoding [Ala^{31}]IGF-I was constructed by replacing the *Bst*EII/*Xba*I fragment of pJY2 (encoding wild-type hIGF-I) (21) with a synthetic DNA fragment formed by the annealing of two oligonucleotides 5′ GTGACAGAGG GTTCCTCTTC AACAAGCCAA CTGGTGCTGG TTCTTCTT 3′ and 5′ CTAGAAGAAG AACCAGCACC AGTT-GGCTTG TTGAAGTAGA ACCCTCT 3′ as described previously (17). These oligonucleotides were designed to form a *Bst*EII/*Xba*I fragment with a mismatch coding for both [Leu^{24},Ala^{31}]IGF-I and [Ala^{31}]IGF-I. The codon for Arg^{21} was altered to destroy the *Bst*EII site in the mutant genes to aid their identification. The codon for Gly^{22} was altered to allow NlaIV digestion to discriminate between the two genes. After two rounds of transformation into *E. coli* DH5, a clone designated pJY31 was identified by NlaIV digestion and confirmed by DNA sequencing. The double mutation, [Leu^{24},Ala^{31}] IGF-I, was not obtained by this technique.

Plasmid pJY168 encoding [Leu^{24},Ala^{31}]IGF-I was constructed by replacing the *Bst*EII/*Xba*I fragment of pJY2 with a synthetic fragment formed by the annealing of two oligonucleotides 5′ GTG-ACAGAGG GTTCCTCTTC AACAAGCCAA CTGGTGCTGG TTCTTCTT 3′ and 5′ CTAGAAGAAG AACCAGCACC AGTT-GGCTTG TTGAAGAGGA ACCCTCT 3′. After two rounds of trans-

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[1] The abbreviation used is: hIGF-I, human insulin-like growth factor I.

formation into *E. coli* DH5 a clone designated pJY168 was identified by *Bst*EII digestion and confirmed by DNA sequencing.

Plasmid pJY60 encoding [Leu^{60}]IGF-I was constructed by replacing the *Xho*I/*Bam*HI fragment of pJY2 with a synthetic fragment formed by the annealing of two oligonucleotides 5′ TCGAAATGCT CTGCGCACCG CTGAAACCGG CTAAATCTGC TTGATAAG 3′ and 5′ GATCCTTATC AAGCAGATTT AGCCGGTTTC AGCGG-TGCGC AGAGCATT 3′. The codon for Glu^{56} was changed from GAG to GAA to destroy the *Xho*I site in the mutant to aid in its identification. After two rounds of transformation into *E. coli* DH5, a clone designated pJY60 was identified by *Xho*I digestion and confirmed by DNA sequencing.

Plasmid pHY91 encoding [Ala^{31},Leu^{60}]IGF-I was constructed by replacing the *Xba*I/*Eco*RI fragment of pJY31 with the *Xba*I/*Eco*RI fragment of pJY60. Clones were identified by *Xho*I digestion and confirmed by DNA sequencing. Plasmid pJY197 encoding [Leu^{24},Leu^{60}]IGF-I was constructed by replacing the *Xba*I/*Eco*RI fragment of pJY137 with the *Xba*I/*Eco*RI fragment of pJY60. Plasmid pJY223 encoding [Leu^{24},Ala^{31},Leu^{60}]IGF-I was constructed by replacing the *Xba*I/*Eco*RI fragment of pJY168 with the *Xba*I/*Eco*RI fragment of pJY60.

Expression and Purification of Mutant IGF-I Peptides—The mutant peptides were produced in *Saccharomyces cerevisiae* strain BJ1995, a gift of Dr. Elizabeth Jones, Carnegie Mellon University. The peptides were purified from the conditioned media in three steps as described previously (21), and their mass was determined by amino acid analysis.

Characterization of the Biological Activities of Mutant IGF-I Peptides—Receptor and binding protein assays were performed as described previously (11, 21). Briefly, type 1 IGF receptor affinity and insulin receptor affinity were determined by measuring the ability of peptides to inhibit the specific binding of ^{125}I-IGF-I (50–80 Ci/g, 0.25 nM) or ^{125}I-insulin (receptor grade, Du Pont-New England Nuclear, 0.6 mCi/ml), respectively, to human placental membranes. Type 2 receptor affinity was measured by determining the ability of peptides to inhibit the specific binding of ^{125}I-MSA (30–50 Ci/g, 0.3 nM) to rat liver membranes. Serum binding protein affinity was determined by measuring the ability of peptides to inhibit the specific binding of ^{125}I-IGF-I (0.3 nM) to acid-treated human serum. Nonspecific binding to the receptor and binding protein preparations was determined by adding excess unlabeled ligand and was <5% of total binding for all assays.

DNA synthesis in L7 fibroblasts was performed as previously described (22), with the following modifications. Cells (8×10^4/well), were seeded onto a 96-well microtiter plate. After 24 and 48 h the media were replaced with media containing 0.1% calf serum instead of 10% calf serum. After 72 or 80 h, peptides or [^{3}H]thymidine were added, respectively. Cells were washed and digested with NaOH at 96 h.

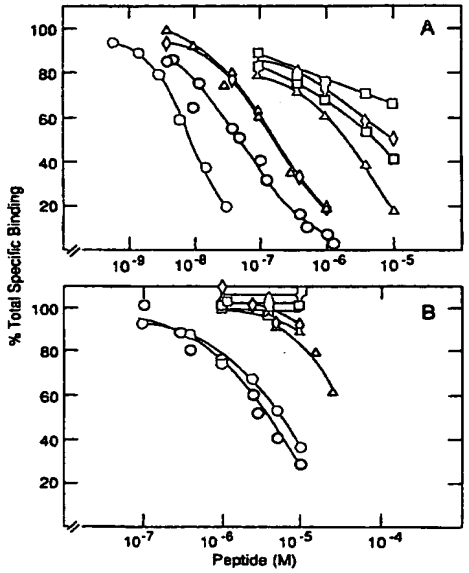

FIG. 1. **Inhibition of ligand binding to human placental type 1 IGF receptors (*A*) or human insulin receptors (*B*) by IGF-I (○), [Ala^{31}]IGF-I (○), [Leu^{24}]IGF-I (△), [Leu^{60}]IGF-I (◇), [Ala^{31},Leu^{60}]IGF-I (□), [Leu^{24},Ala^{31}]IGF-I (△), [Leu^{24},Leu^{60}] IGF-I (◇), and [Leu^{24},Ala^{31},Leu^{60}]IGF-I (□).** Data are expresed as the percent of maximal specific binding determined in the absence of added peptide. Each point represents the mean of two independent determinations.

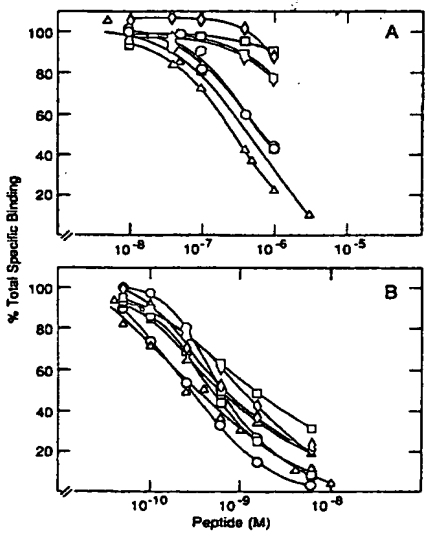

FIG. 2. **Inhibition of ligand binding to rat liver type 2 IGF receptors (*A*) or acid-stable human serum binding proteins (*B*) by IGF analogs.** Data are expressed as the percent of maximal specific binding determined in the absence of added peptide. Each point represents the mean of two independent determinations. The analogs are as defined in the legend to Fig. 1.

RESULTS

Human IGF-I analogs with single, double, or triple point substitutions for the 3 tyrosines at positions 24, 31, and 60 were produced by expression of mutated IGF-I genes in yeast as described under "Experimental Procedures." The analogs were purified from conditioned media in three steps. The purity of the proteins was confirmed using sodium dodecylsulfate-polyacrylamide gel electrophoresis as described previously (17). The final yields of analogs from 1 liter of yeast-conditioned media were 444 μg for [Ala^{31}]IGF-I, 291 μg for [Leu^{60}]IGF-I, 224 μg for [Leu^{24},Ala^{31}]IGF-I, 396 μg for [Leu^{24},Leu^{60}]IGF-I, 218 μg for [Ala^{31},Leu^{60}]IGF-I, and 306 μg for [Leu^{24},Ala^{31},Leu^{60}]IGF-I.

The single point mutations of hIGF-I, [Leu^{24}]IGF-I, [Ala^{31}] IGF-I, and [Leu^{60}]IGF-I, result in a 18-, 6-, or 20-fold loss in affinity, respectively, for the human placental type 1 IGF receptor (Fig. 1*A*). Double mutations, [Leu^{24},Ala^{31}]IGF-I, [Leu^{24},Leu^{60}]IGF-I, and [Ala^{31},Leu^{60}]IGF-I, result in a 240-, 1200-, or 520-fold loss in affinity, respectively, at the type 1 receptor. The analog in which all three tyrosines are replaced, [Leu^{24},Ala^{31},Leu^{60}]IGF-I has little affinity for the type 1 receptor.

Analysis of the analogs' ability to bind to the human placental insulin receptor indicates that [Ala^{31}]IGF-I is equipotent to hIGF-I at the insulin receptor and [Leu^{24}]IGF-I demonstrates a 5-fold loss in affinity (Fig. 1*B*). [Leu^{60}]IGF-I and [Leu^{24},Ala^{31}]IGF-I demonstrate weak binding; and the other multiple mutation analogs are inactive at the insulin receptor at concentrations up to 10 μM. All of the analogs appear to maintain high affinity for the acid-stable human serum binding proteins (Fig. 2*B*). Analogs [Leu^{24}]IGF-I, [Ala^{31}]IGF-I,

TABLE I

Effects of tyrosine replacements in hIGF-1 on binding to the human placental type 1 IGF receptor, the rat liver type 2 IGF receptor, the human placental insulin receptor (IR), and human serum binding proteins (hBP).

Peptide	IC_{50}*			
	Type 1	Type 2	IR	hBP
	nM	*μM*	*μM*	*nM*
hIGF-I	8.7 ± 1.1	0.7 ± 0.2	5.7 ± 0.1	0.6 ± 0.1
[Leu^{24}]IGF-I	160 ± 40	0.9 ± 0.6	29 ± 4	0.4 ± 0.1
[Ala^{31}]IGF-I	51 ± 10	0.8 ± 0.2	3.6 ± 0.1	0.4 ± 0.1
[Leu^{60}]IGF-I	170 ± 10	>1	>10	0.7 ± 0.01
[Ala^{31}, Leu^{60}]IGF-I	5,600 ± 440	>1	>10	0.5 ± 0.01
[Leu^{24}, Ala^{31}]IGF-I	2,000 ± 10	0.3 ± 0.1	>10	0.6 ± 0.01
[Leu^{24}, Leu^{60}]IGF-I	10,000	>1	>10	1.1 ± 0.1
[Leu^{24}, Ala^{31}, Leu^{60}]IGF-I	>10,000	>1	>10	1.5 ± 0.2
hIGF-II	30	0.003		
Insulin	500	>10	0.003	>10,000

* Concentration for half-maximal inhibition of ligand binding, mean ± S.D., n = at least 2.

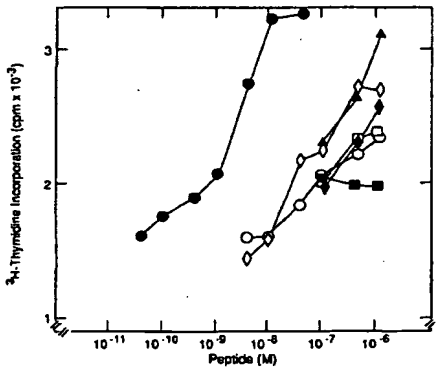

FIG. 3. **Stimulation of DNA synthesis in L7 murine fibroblasts by IGF analogs.** Data points were determined in quadruplicate. Relative potencies were determined using the parallel line bioassay technique (23). The analogs are as defined in the legend to Fig. 1.

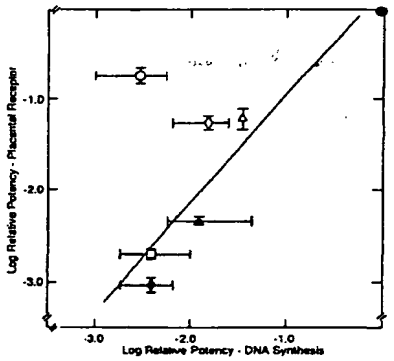

FIG. 4. **Comparison of the relative potencies of IGF analogs at the type 1 IGF receptor and in DNA synthesis.** The analogs are as defined in the legend in Fig. 1. The *line* represents the linear regression analysis (correlation coefficient = 0.93) of the data points excluding [Ala^{31}]IGF-I (O). *Vertical error bars* reflect the standard deviation in relative affinity for receptor as given in Table I. *Horizontal error bars* reflect the 95% confidence limits in the calculation of relative potency of the data in Fig. 3.

and [Leu^{24},Ala^{31}]IGF-I are equipotent to hIGF-I at the rat liver type 2 IGF receptor, whereas all analogs containing Leu^{60} demonstrate little measurable affinity at this receptor (Fig. 2*A*). Table I summarizes the affinities of these analogs for the

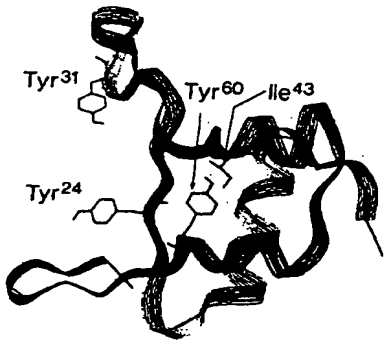

FIG. 5. **The proposed structural model of IGF-I highlighting tyrosines 24, 31, and 60 and isoleucine 43.**

receptors and binding proteins.

The IGF analogs with single and double point mutations stimulate DNA synthesis in L7 murine fibroblasts (Fig. 3). All of the analogs are less potent than IGF-I. [Leu^{24},Ala^{31}, Leu^{60}]IGF-I is inactive at concentrations up to 1 μM. The relative potencies of these analogs were calculated using the parallel line bioaassay technique (23). Fig. 4 shows the relationship between the relative potency of the analogs at the human placental type 1 IGF receptor and their ability to stimulate DNA synthesis in L7 fibroblasts. With the exception of [Ala^{31}]IGF-I (*open circles* in Fig. 4), this relationship is linear.

DISCUSSION

Previous data from our laboratories have shown that distinct regions of hIGF-I are responsible for maintaining high affinity binding to the types 1 and 2 IGF receptors and soluble acid-stable serum binding proteins (10–13). For example, replacement of residues 49–51 and 55,56 in the A region with the analogous residues in the A chain of insulin selectively modulates binding to type 2 IGF receptors (12). Replacement of residues 3,4 and 15,16 in the B region of IGF-I results in a dramatic loss in affinity for acid-stable human serum binding proteins with no change in affinity for the types 1 and 2 IGF receptors (11).

Characterization of naturally occurring mutants of insulin with reduced affinity for the insulin receptor demonstrates that the phenylalanine at B25 and the tyrosine at position A19 are important in maintaining high affinity binding of insulin to its receptor (14, 24). Previous work from our laboratories demonstrated that the analogous residue to B25 of

insulin, Tyr^{24} is important in maintaining the high affinity of hIGF-I to the type 1 IGF receptor (10). In addition, replacement of residues 28–37 of hIGF-I with a 4-glycine bridge reduces the ability of hIGF-I to bind the type 1 receptor (13). The data presented here demonstrate that at least part of the loss in receptor affinity of this analog is due to the loss of Tyr^{31}.

Blundell and co-workers (25, 26) have proposed a three-dimensional model of hIGF-I based on its structural homology with insulin. This model, shown in Fig. 5, predicts that the hydroxyl groups in Tyr^{24} and Tyr^{31} are 10 Å apart and are positioned at the surface of the molecule. We propose that these 2 residues are important for the interaction of hIGF-I with the type 1 receptor; thus [Leu^{24},Ala^{31}]IGF-I has 240-fold reduced affinity for the type 1 IGF receptor. The retention of high affinity binding to acid-stable human serum binding proteins and to the type 2 IGF receptor suggests that the loss of affinity for the type 1 receptor is specific and probably not due to dramatic changes in the overall conformation of the analog.

The loss of Tyr^{31} has no affect on the binding of [Ala^{31}] IGF-I to the insulin receptor (Fig. 1*B*). This is consistent with the absence of an analogous residue in mature insulin. These data support our hypothesis that the type 1 IGF receptor and the insulin receptor recognize distinct but overlapping domains of their respective ligands (27). Thus, replacement of residues 24 (10), 31, or 28–37 (13) result in loss of type 1 receptor binding, while replacement of residues 24 (10), 15–16 (11), or the deletion of 63–70 (13) modify insulin receptor binding.

Nakagawa and Tager (28) have proposed that the loss of insulin receptor affinity with replacement of Phe^{B25} of insulin is due to main chain interactions with the insulin receptor and not due to side chain involvement in binding affinity. We have no direct evidence that the changes we have made at residues 24 and 31 do not result in moderate conformational changes in this region. However, we have shown that changing residues 1–16 or 42–56 of hIGF-I to the analogous residues of insulin has no effect on binding to the type 1 IGF receptor (11, 12). These modifications have altered the characteristics of nearly every surface region in hIGF-I. These data strongly suggest that residues between positions 24–37 of hIGF-I comprise the important domain in type 1 receptor interaction. We cannot conclude from our data whether the changes in affinity upon replacement of residues Tyr^{24} and/or Tyr^{31} are due to side chain or main chain interactions with the type 1 IGF receptor.

The third tyrosine in hIGF-I is in an analogous position to the tyrosine at A19 in insulin. Studies using the accessibility of the tyrosine residues to iodination as a measure of binding domains suggests that all three tyrosines of hIGF-I are involved in type 1 IGF receptor binding (15). Our data show that the replacement of Tyr^{60} results in loss of affinity for both the types 1 and 2 IGF receptors; all analogs missing this residue show reduced affinity for both receptors. Previous data from insulin indicates that Tyr^{A19} and Ile^{A2} are close enough for van der Waals interaction and that this interaction may stabilize the A chain of insulin. Mutations in insulin of either A19 or A2 result in a dramatic loss of insulin receptor binding. Similarly, the proposed model for hIGF-I predicts that Ile^{43} and Tyr^{60} are 3.5 Å apart. We have shown previously that determinants in the A region of hIGF-I are required for binding to the type 2 IGF receptor and to the human amniotic fluid binding protein, IGF binding protein 1 (29). A perturbation of the A region of hIGF-I due to the loss of the Tyr^{60}/Ile^{43} interaction may thus affect indirectly both type 2 receptor binding and IGF binding protein 1 binding. In fact, analogs in which Tyr^{60} has been replaced with leucine have reduced affinity for a 30-kDa IGF binding protein from BalbC/3T3 cells (data not shown) but normal affinity for human serum binding proteins. The model predicts that the tyrosine hydroxyls at positions 24 and 31 are 15 Å and 19 Å, respectively, from the hydroxyl of Tyr^{60} (Fig. 5). However, the model also predicts that the side chain of Tyr^{60} faces inward toward the core of the molecule. Therefore, the effect of the absence of Tyr^{60} on type 1 IGF receptor binding may also be due to moderate conformational changes in the receptor binding region.

With the exception of [Ala^{31}]IGF-I, the potencies of these analogs in stimulating DNA synthesis in L7 cells closely correlates with their affinities for the human placental type 1 IGF receptor. [Ala^{31}]IGF-I has 6-, 16-, 16-, 10-, and 40-fold lower affinity than IGF I at the type 1 receptor from human placenta, sheep placenta, rat placenta, dog placenta, and L7 cells, respectively (data not shown). However, these species or tissue differences in receptor affinity for [Ala^{31}]IGF-I do not totally account for the 300-fold loss in potency in L7 cells. This analog is 100- and 13-fold less potent than IGF I in stimulating DNA synthesis in BalbC/3T3 cells and rat A10 cells, respectively (data not shown). Thus, in these latter two cell lines, its potency more nearly reflects its affinity for type 1 receptors. At the present time it is unclear if the unexpected loss in potency in L7 cells is due to lower intrinsic activation of the receptor in L7 cells, increased sensitivity to proteolytic degradation, or other reasons. Excess [Ala^{31}]IGF-I (100 nM) did not inhibit the ability of IGF-I (0.1–10 nM) to stimulate DNA synthesis in L7 cells (data not shown).

In conclusion, these studies have defined further the region of hIGF-I responsible for high affinity binding to the type 1 IGF receptor. These analogs should also be useful tools for determining the relative roles of receptor and binding proteins in modulating IGF-I biological activity.

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Pharmacology

Displacement of insulin-like growth factors from their binding proteins as a potential treatment for stroke

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ABSTRACT **Insulin-like growth factors I and II (IGF-I and IGF-II) play an important role in normal growth and brain development and protect brain cells from several forms of injury. The effects of IGFs are mediated by type-I and type-II receptors and modulated by potentially six specific binding proteins that form high-affinity complexes with IGFs in blood and cerebrospinal fluid (CSF) and under most circumstances inactivate them. Because brain injury is commonly associated with increases in IGFs and their associated binding proteins, we hypothesized that displacement of this large "pool" of endogenous IGF from the binding proteins would elevate "free" IGF levels to elicit neuroprotective effects comparable to those produced by administration of exogenous IGF. A human IGF-I analog [($Leu^{24,59,60}$, Ala^{31})hIGF-I] with high affinity to IGF-binding proteins (K_i = 0.3–3.9 nM) and no biological activity at the IGF receptors (K_i = >10,000 nM) increased the levels of "free, bioavailable" IGF-I in the CSF. Intracerebroventricular administration of this analog up to 1h after an ischemic insult to the rat brain had a potent neuroprotective action comparable to IGF-I. This novel strategy for increasing "free" IGF levels in the brain may be useful for the treatment of stroke and other neurodegenerative diseases.**

Insulin-like growth factors I and II (IGF-I and IGF-II) are multifunctional peptides essential for normal growth and development (1). Their biological actions are mediated by the type-I IGF receptor (2) and possibly the type-II IGF receptor, which is identical to the cation-independent mannose 6-phosphate receptor (3). In the circulation and interstitial fluids, including the cerebrospinal fluid (CSF), IGFs are almost entirely associated with one or more of at least six IGF-binding proteins (IGFBPs) that bind IGFs with high affinity, thus limiting their interaction with receptors, and potentially providing a "reservoir" of biologically inactive IGF (1). IGFs undoubtedly play an important role in brain development and may also be important after injury. IGF treatment protects the developing or adult brain from hypoxic-ischemic injury (4–7) and forebrain ischemia (8), induces myelination (9–11), and reduces neuronal death *in vitro* caused by diverse forms of injury (12–16). Paradoxically, injury to the developing or adult brain is commonly associated with increases in brain IGFs as well as their associated binding proteins (4, 17–26). Consequently, even though IGFs are elevated, they may be complexed with their binding proteins and unavailable to provide neuroprotection. The IGF system, therefore, provides a rather unique opportunity for utilizing an endogenous neuroprotective factor. We hypothesized that displacement of the large "pool" of IGF from the IGFBPs in the brain would elevate "free" IGF levels, increasing receptor activation to elicit similar actions to administration of IGF-I itself.

In the present studies, we examined the role of brain IGFs and IGFBPs in neuroprotection by comparing the effects of hIGF-I with the selective, high-affinity IGFBP ligand inhibitor, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I in *in vitro* studies of release of "free" bioactive IGF-I from rat cerebrospinal fluid and in *in vivo* studies to evaluate their neuroprotective effects in a rat model of focal ischemia. Data suggest that IGFBPs, by neutralizing IGFs, may serve to limit the actions of the peptides under both physiological and pathological conditions. Furthermore, the results demonstrating potent neuroprotective effects of the IGFBP ligand inhibitor comparable to IGF-I suggest that this strategy for increasing "free" IGF levels in the brain may be useful for the treatment of stroke and other neurodegenerative diseases.

MATERIALS AND METHODS

Synthesis and Purification of Peptides. hIGF-I and hIGF-II were obtained from Sigma. [Nle^{29}]hIGF-I and [$Leu^{24,59,60}$, Ala^{31}]hIGF-I were synthesized by a solid-phase peptide synthesis procedure as described previously (27) by using a t-butoxycarbonyl-Ala-(oxymethyl)-phenylacetamidomethyl (PAM) resin on a Beckman 990 peptide synthesizer. Derivatized amino acids and resin used in the synthesis were purchased from Bachem. After the last residue was coupled onto the growing peptide chain, the protected peptide resin was treated with the low–high hydrogen fluoride cleavage procedure (28) to remove the peptide from the resin anchor and deprotect the side-chain functional groups. The crude peptide was extracted with 5 M guanidine HCl in 0.1 M NH_4OAc, and the pH of the extract was maintained at 5 with HOAc. After filtering off the resin, the solution was diluted with 0.1 M NH_4OAc to 2 M guanidine HCl to a peptide concentration of ≈1 mg/ml. The peptide was cyclized by air oxidation by stirring at room temperature for 24 h while maintaining the pH at 8.4 with 10% concentrated NH_4OH. After oxidation, the pH was adjusted to 5 and the solution was dialyzed against 0.1 M acetic acid at room temperature to remove the guanidine salt. The recovered dialysate was lyophilized and the crude product was purified by gel filtration on Sephadex G-50F, followed by carboxymethyl cellulose cation-exchange chromatography and preparative HPLC on a KP-100 Gradient HPLC system with a Vydac C18 cartridge (Biotage, Charlottesville, VA). The purified product was verified by mass spectrometric analysis on a SCIEX/AP1 LC/MS system equipped with an ion-spray source (Perkin–Elmer).

Radioligand Binding Assay. Human IGFBP-1, BP-4, and BP-5 were expressed in the BaculoGold Expression System (PharMingen) in Sf9 insect cells and purified by affinity

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Abbreviations: IGF, insulin-like growth factor; IGFBP, IGF binding protein; CSF, cerebrospinal fluid.

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chromatography on a hIGF-I-coupled Affi-Gel 10 column, followed by reverse-phase HPLC. Human IGFBP-2 and BP-3 were isolated from outdated plasma as described previously (29). The binding assay was performed at room temperature in duplicate in 0.02% Nonidet P-40/PBS buffer, pH 7.2. Two hundred microliters of a 2.5 nM IGFBP solution (0.5 pmol) was added to a 12 × 75-mm glass test tube. The reaction was started by the addition of 100 μl buffer, hIGF-I, hIGF-II, or [$Leu^{24,59,60}$, Ala^{31}]hIGF-I solution, followed by 100 μl of [^{125}I]hIGF-I (30,000 cpm, specific activity ≈2,200 Ci/mmol; New England Nuclear). After incubation for 2 h, 100 μl of 20% BSA and 500 μl of 20% PEG-8000 in the PBS buffer were added and the mixture was vortexed and then centrifuged for 30 min at 3,000 rpm. The supernatant was carefully removed by suction and the pellet was counted in a γ-counter.

Radioligand and Western Blot Analysis of the IGF-Binding Proteins. Twenty microliters of rat CSF was fractionated by SDS/PAGE and blotted onto nitrocellulose paper, and the blot was then incubated with [^{125}I]hIGF-I and examined by autoradiography according to the procedure described previously (30). Western blot analysis of the IGFBPs was performed by electrophoresing 20 μl of rat CSF per lane on SDS/PAGE, followed by blotting of the gel onto nitrocellulose paper, according to the published procedure (30). The nitrocellulose paper was then cut into replicate strips, and one strip was incubated with IGFBP-2 antiserum (Upstate Biotechnology, Lake Placid, NY) whereas the other was incubated with IGFBP-5 antiserum raised in a rabbit with a synthetic peptide fragment as described previously (30). The stained bands were revealed by incubation with peroxidase-conjugated goat anti-rabbit IgG, followed by chemiluminescence detection with a commercial kit (Pierce).

Gel Filtration Analysis of Dissociated IGF-I from the IGF-I/IGF-Binding Protein Complex. Five hundred microliters of rat CSF was incubated with [^{125}I]hIGF-I at 37°C for 1 h to incorporate the radioiodinated peptide into the complex, and the incubated fluid was divided into 100-μl aliquots. To each aliquot was added buffer (control), IGF-I, or [$Leu^{24,59,60}$, Ala^{31}]hIGF-I, and the mixture was incubated for 1 h at 37°C, followed by storage on ice. For gel-filtration analysis, each aliquot was diluted with 400 μl 0.02% NaN_3/0.1% BSA/PBS buffer and the diluted sample was loaded onto a 1 × 50 cm Sephadex G-50F column; the column was developed with the same buffer at a flow rate of 0.5 ml/min at room temperature. The collected fractions were counted in a γ-counter.

Fibroblast Proliferation Assay. Biological activities of hIGF-I, [Nle^{59}]hIGF-I, and the IGFBP ligand inhibitor [$Leu^{24,59,60}$, Ala^{31}]hIGF-I were tested in a BALB/c 3T3 fibroblast assay (31). The ability of the peptides to induce proliferation was measured by counting the amount of [^{3}H]thymidine incorporated by the cells. Cells were aliquoted to 96-well microtiter plates (180 μl per well). After a 48-h incubation at 37°C and 5% CO_2, the plates were washed twice with 0.1% calf serum/DMEM and incubated for an additional 24 h. Twenty microliters of sample and 1 μCi [^{3}H]thymidine (New England Nuclear) were added to each well, and the plates were incubated for a further 24 h. After incubation, the medium was removed and the cells were fixed by the addition of 200 μl of a 25% acetic acid/75% ethanol solution per well. After removal of the fixing solution, the plates were washed three times with cold 10% trichloroacetic acid and the cells were lysed in 200 μl 0.2 M NaOH. The entire 200 μl of lysate solution was transferred into a scintillation vial; 2.5 ml of scintillation liquid was added and the vials were counted in a γ-counter.

Rat Middle Cerebral Artery Occlusion Model of Focal Ischemia. Male Sprague–Dawley rats (Charles River) were housed in a 12-h light/12-h dark cycle and allowed food and water *ad libitum*. The experiment protocol was approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Rats weighing 160–190 g were anesthetized with isoflurane (4% induction, 2.2% maintenance) in O_2, and indwelling guide cannulae were stereotaxically implanted in the right, lateral ventricle [coordinates in mm: lateral (+1.5); anteroposterior (−0.8); dorsoventral (−3.0), relative to Bregma] to permit subsequent injections into the CSF. Ten days later, rats were reanesthetized with halothane (4% induction, 2–2.5% maintenance) in O_2, and focal cerebral ischemia was induced by permanent occlusion of the left, middle cerebral artery (MCAo) proximal to the lenticulostriate branch, by electrocoagulation. Throughout surgery and recovery from anesthesia, animals were maintained normothermic by means of a heated blanket. Peptides or vehicle (sterile water) were injected into the lateral ventricle (in a volume of 5 μl) over 2–3 min, either concurrent with or 1 h after MCAo. One day after MCAo, animals were killed and the brains were removed. Delineation of the lesion was determined on fresh 500-μm coronal brain sections incubated in 2% tri-phenyl-tetrazolium chloride (TTC; Sigma) by using an indirect approach thus "correcting" for any swelling. Lesion volume was calculated for each brain by integration of the areas of infarct in each section. These procedures are described in more detail elsewhere (32). Peptide-treated groups were compared with vehicle-treated animals by using Student's unpaired *t* test.

RESULTS AND DISCUSSION

Before this study, Bayne *et al.* (33) had reported that an hIGF-I analog, [$Leu^{24,60}$, Ala^{31}]hIGF-I, has a >1,200-fold loss in affinity to the type-I IGF receptor and little measurable affinity for the type-II receptor. To facilitate the synthesis and stability of this analog, the endogenous methionine at position 59 was replaced with leucine. The resulting compound, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I, was tested with hIGF-I and hIGF-II for displacement of [^{125}I]hIGF-I binding to human IGFBP-1, -2, -3, -4, and -5, and biological activity in BALB/c 3T3 fibroblast cells, which proliferate in response to IGFs. As shown in Table 1, hIGF-I and hIGF-II have comparable or somewhat higher affinities for IGFBP-1, -2, -3, -4, and -5 (K_i values = 0.01–0.22 nM) than for their homologous type-I and type-II receptors (K_i values = 1.5 and 0.2 nM, respectively). In contrast to the relative lack of selectivity of IGF-I and IGF-II between the IGFBPs and IGF receptors, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I has high affinity for IGFBP-1, -2, -3, -4, and -5 (K_i values = 0.28–3.91 nM) and is inactive at IGF receptors (K_i values = >10,000 nM) (Table 1). Furthermore, in contrast to hIGF-I, which dose-dependently stimulated

Table 1 Relative affinity and selectivity of IGF-I, IGF-II, and IGFBP ligand inhibitor (IGFBP-LI) for IGF-binding proteins and the type-I and type-II IGF receptors

Protein or receptor	K_i, nM		
	IGF-I	IGF-II	IGFBP-LI
IGFBP-1	0.12 ± 0.03	0.051 ± 0.008	1.91 ± 0.10
IGFBP-2	0.06 ± 0.01	0.010 ± 0.005	1.92 ± 0.80
IGFBP-3	0.21 ± 0.04	0.023 ± 0.005	1.80 ± 0.20
IGFBP-4	0.10 ± 0.03	0.032 ± 0.004	0.28 ± 0.10
IGFBP-5	0.22 ± 0.04	0.040 ± 0.004	3.91 ± 2.40
Type-I receptor	1.5	3.0	>10,000
Type-II receptor	400	0.2	>10,000

The relative affinities of hIGF-I, hIGF-II, and the IGFBP-LI, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I for the various IGFBPs were determined in radioligand-binding assays as described in *Materials and Methods*. Data represent the mean ± SEM of three separate determinations. The affinity constants of the IGF-I and IGF-II for the type-I and type-II receptors were taken from ref. 34 and 35, respectively, whereas the affinity constants of the IGFBP ligand inhibitor for the IGF receptors were taken from ref. 33.

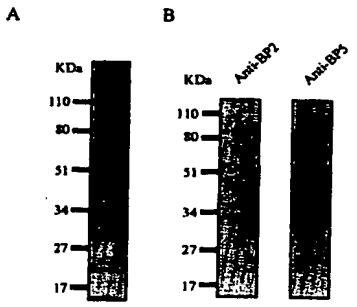

FIG. 1. Identification of IGF-binding proteins in rat cerebrospinal fluid. (*A*) Radioligand blot of IGF-binding proteins present in rat cerebrospinal fluid. Note that the major radiolabeled band detected has a molecular mass of ≈32 kDa, which corresponds to the molecular mass of IGFBP-2 or IGFBP-5. (*B*) Western blot identifying the major IGFBP in rat CSF as BP-2.

DNA synthesis in 3T3 fibroblasts with an IC_{50} of 5–10 nM, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I had no activity in the assay at concentrations of up to 8 μM, indicating a lack of interaction with the IGF receptors in this functional assay.

We evaluated the ability of the IGFBP ligand inhibitor, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I, to displace the bound IGF-I and elevate "free" biologically active levels of the peptide in rat CSF and in the BALB/c 3T3 fibroblast proliferation assays. In agreement with previous reports (36, 37), using ligand and Western blot analyses, we determined that the most abundant IGFBP in rat CSF is BP-2 (Fig. 1 *A* and *B*, respectively). Gel filtration analysis of rat CSF that had been preincubated with trace quantities of [^{125}I]hIGF-I demonstrated that ≈64% of [^{125}I]hIGF-I eluted as a higher molecular mass complex (presumably bound to IGFBP-2) and ≈36% eluted at a molecular mass corresponding to "free" [^{125}I]hIGF-I (Fig. 2). Incubation of the [^{125}I]hIGF-I-incorporated CSF with either IGF-I (0.1 μM) or [$Leu^{24,59,60}$, Ala^{31}]hIGF-I (1 μM) resulted in a decrease in the proportion of IGF-I/IGFBP complex and a corresponding increase in "free" [^{125}I]hIGF-I levels (Fig. 2). The higher concentration of the IGFBP ligand inhibitor than IGF-I required to increase "free" IGF-I levels is in keeping with the ≈10- to 20-fold lower affinity of the IGFBP ligand inhibitor for IGFBPs than IGF-I itself (Table 1). The ability of the IGFBP ligand inhibitor to release bioactive IGF-I was further evaluated in the 3T3 fibroblast assay. Human IGF-I (3 nM) produced robust proliferation of 3T3 fibroblasts as reflected by increased [^{3}H]thymidine incorporation; the hIGF-I-induced proliferation was substantially blocked by addition of 20 nM IGFBP-2 (Fig. 3). The addition of [$Leu^{24,59,60}$, Ala^{31}]hIGF-I dose-dependently reversed the neutralizing effect of IGFBP-2 on IGF-I (ED_{50} = 200 nM), demonstrating the ability of the IGFBP ligand inhibitor to displace hIGF-I bound to IGFBP-2 (Fig. 3). Overall, these *in vitro* data clearly demonstrate that the IGFBP ligand inhibitor is capable of interacting with the binding protein in a specific manner to displace complexed IGF-I and release "free" bioactive peptide.

In view of the potent neuroprotective and regenerative effects of IGFs (4–16), we tested the hypothesis that displacement of IGF from its BPs in the brain could confer neuroprotection in a clinically relevant model of stroke in the rat. Although the models of ischemia [hypoxic-ischemia (4–7) and forebrain ischemia (8)] previously used to evaluate the effects of IGF-I provide important information on the effects of ischemia on the brain, they are not considered as models of stroke. It is generally accepted that occlusion of a single intracranial artery (the middle cerebral artery, MCA) provides the best model to study stroke (38). Because of the relatively large quantities of IGF-I required for *in vivo* studies, synthetic [Nle^{59}]hIGF-I was employed. In [Nle^{59}]hIGF-I the endogenous methionine at position 59 is replaced by the isosteric norleucine to eliminate the possibility of oxidizing the methionine to methionine sulfoxide during cyclization of the three disulfide bonds by air oxidation in the synthesis of the molecule. Bioassay of [Nle^{59}]hIGF-I and hIGF-I in BALB/c 3T3 fibroblasts showed no difference in the proliferative potency between the two compounds. Adult male rats previously implanted with lateral cerebral ventricular guide cannula were subjected to ischemia by permanent occlusion of the MCA (MCAo), and the resulting brain lesion was visualized and quantified 24 h later. Animals that had received a single intracerebroventricular (i.c.v.) injection of synthetic

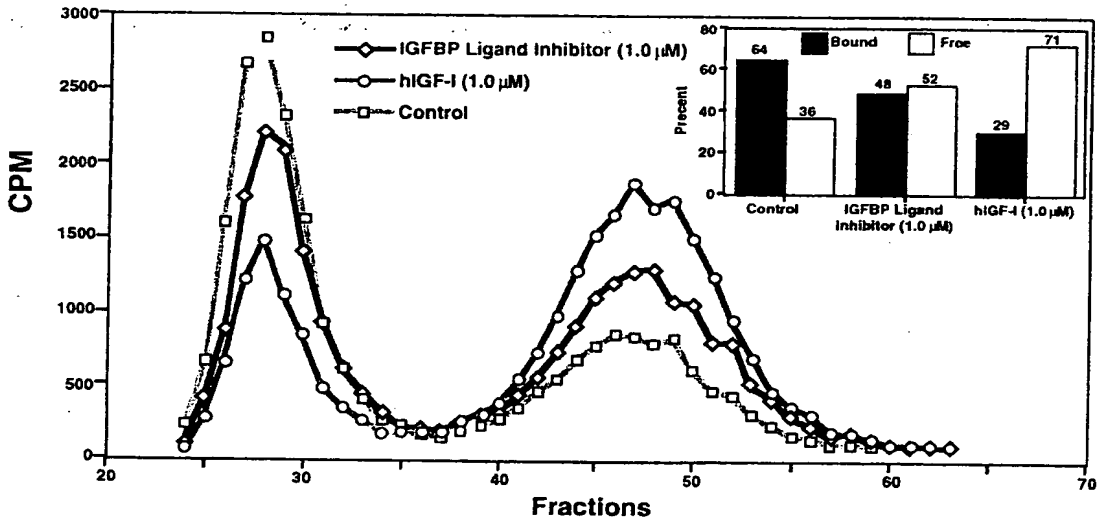

FIG. 2. Gel-filtration analysis of rat CSF showing the relative proportions of bound and "free" [^{125}I]hIGF-I dissociated by IGF-I or the IGFBP ligand inhibitor. The Sephadex G-50F gel-filtration profiles of rat CSF in the absence (Control) or presence of hIGF-I [hIGF-I (0.1 μM)] or 1.0 μM of [$Leu^{24,59,60}$, Ala^{31}]hIGF-I [IGFBP Ligand Inhibitor (1.0 μM)] are shown. The quantified data representing the relative proportions of the hIGF-I/IGFBP complex (Bound) and "free" hIGF-I (Free) are presented in the *Inset*.

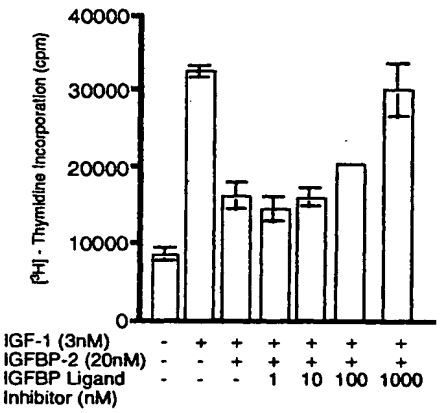

FIG. 3. Reversal of IGFBP-2 inhibition of hIGF-I-stimulated fibroblast proliferation by [Leu^{24,59,60}, Ala^{31}]hIGF-I *in vitro*. Human IGF-I dose-dependently stimulated DNA synthesis with an ED_{50} of 5–10 nM. In contrast, [Leu^{24,59,60}, Ala^{31}]hIGF-I did not induce DNA synthesis in 3T3 cells at any of the doses tested (0.1–8,000 nM). IGFBP-2 (20 nM) substantially inhibited the proliferative effect of 3 nM hIGF-I. Addition of IGFBP ligand inhibitor dose-dependently reversed this inhibition with an ED_{50} of 200 nM.

[Nle^{59}]hIGF-I (50 μg) or the IGFBP ligand inhibitor (50 μg) at the time of MCAo had much smaller total lesion volumes than those injected with vehicle, primarily because of a reduction of the cerebral cortical infarct volume, although some protection was also evident in the striatum (Fig. 4*A*). The extent of neuroprotection (40–50%) was comparable for [Nle^{59}]hIGF-I and the IGFBP ligand inhibitor and is in keeping with that seen after treatment with NMDA receptor antagonists (39, 40). Remarkably, the extent of protection was similar whether [Nle^{59}]hIGF-I or [Leu^{24,59,60}, Ala^{31}]hIGF-I was administered concurrent with (0 h) or 1 h after occlusion of the artery (Fig. 4*B*), providing a therapeutic window for the treatment as is available in this rat model of ischemia. In the more slowly developing hypoxic-ischemia model, IGFs confer neuroprotective effects when administered up to 2 h after the insult (4–7). These observations taken together with previous data indicating that i.c.v. injection of 50 μg hIGF-I has no impact on plasma glucose levels or body temperature of ischemic rats (7) suggest that IGFs protect neurons by interfering with the pathological pathways that are initiated after ischemia.

The mechanisms through which the IGFBP ligand inhibitor and IGFs produce their neuroprotective effects are at present unclear. Ischemic neuronal damage has been attributed, in part, to the extracellular accumulation of excitatory amino acids; in preliminary studies done in our laboratory, the IGFBP ligand inhibitor [Leu^{24,59,60}, Ala^{31}]hIGF-I attenuated the loss of pyramidal neurons in the hippocampus after intrahippocampal administration of quinolinic acid. In addition to producing their neuroprotective effects by interfering with endogenous mediators of ischemia such as glutamate, IGFs have the distinct advantage of also having the ability to act as regenerative growth factors. The IGFBP ligand inhibitor is capable of increasing the release of not only IGF-I but also IGF-II, which also has neuroprotective effects (13). The release of IGF-II in addition to IGF-I by the IGFBP ligand inhibitor may represent a therapeutic advantage over IGF-I treatment whose selectivity may be limited to actions at the type-I IGF receptor. Because neurodegeneration may be associated with lower levels of "free" bioactive IGFs, in part, because of increased brain expression of IGFBPs (4, 17–26), displacement of this "pool" of endogenous IGFs from their binding proteins with ligand inhibitors seems appropriate. The increased expression of brain IGFBPs (4, 17–26) may also serve to limit the actions of exogenously administered IGFs and provides strong support for the therapeutic relevance of IGFBP ligand inhibitors for the treatment of neurodegeneration. In addition, because the IGFBP ligand inhibitor approach achieves its effect by elevating local endogenous levels of "free" IGFs, a ceiling effect is reached when all the IGFs are released from IGFBPs, thus limiting the side effects that may occur after global activation of IGF receptors by exogenously administered IGF-I. This advantage is evident in other systems such as the corticotropin-releasing factor (CRF) family of peptides, in which the actions of the endogenous peptide(s) are limited by a CRF-binding protein (41). For example, CRF-binding protein ligand inhibitors, like CRF-receptor agonists, enhance learning and memory (42, 43) and blunt excessive weight gain (44) in a variety of rodent models. However, in

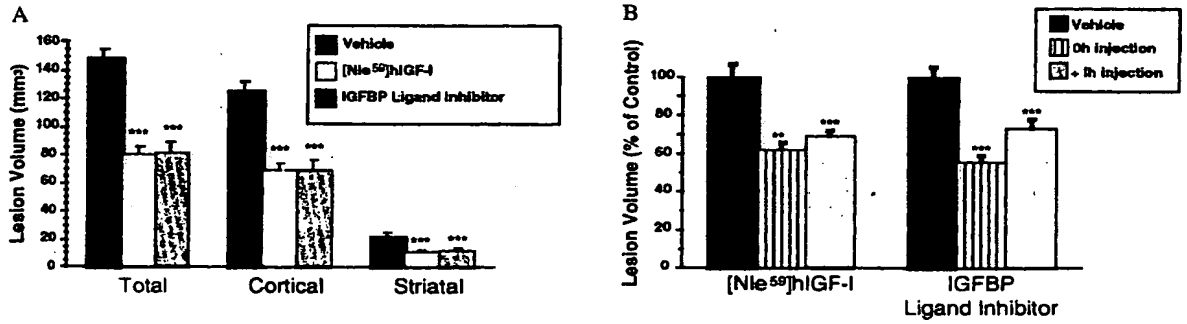

FIG. 4. The protective effects of [Nle^{59}]hIGF-I and the IGFBP ligand inhibitor on ischemic brain damage. (*A*) In the first series of experiments, the effect of concurrent administration of [Nle^{59}]hIGF-I or [Leu^{24,59,60}, Ala^{31}]hIGF-I was determined. Data are presented as mean lesion volume ± SEM. Animals injected at the time of MCAo with [Nle^{59}]hIGF-I (50 μg, n = 7, open bar) or the IGFBP ligand inhibitor, [Leu^{24,59,60}, Ala^{31}]hIGF-I (50 μg, n = 6; gray bar) had dramatically and statistically reduced lesion volumes compared with animals injected with vehicle (n = 6, solid bar). Protection was observed in cerebral cortical and striatal tissue. ***, $P < 0.001$. (*B*) The effects of delaying administration of [Nle^{59}]hIGF-I or the IGFBP ligand inhibitor [Leu^{24,59,60}, Ala^{31}]hIGF-I were determined in a separate series of experiments. Data are presented as the percentage of the mean lesion size of the respective vehicle-treated group (mean ± SEM). As observed in the previous experiment, animals injected at the time of MCAo with [Nle^{59}]hIGF-I (50 μg, n = 7, striped bar) or the IGFBP ligand inhibitor (50 μg, n = 6, striped bar) had dramatically and statistically reduced lesion volumes compared with animals injected with vehicle (n = 6, solid bar). When administration of the peptide was delayed to 1 h after MCAo, protection with [Nle^{59}]hIGF-I (50 μg, n = 7, gray bar) was remarkably similar, and protection with IGFBP ligand inhibitor (50 μg, n = 8, gray bar) was only slightly less than observed with concurrent administration. **, $P < 0.01$; ***, $P < 0.001$.

marked contrast to the effects of a CRF-receptor agonist, CRF-binding protein ligand inhibitors do not induce anxiety (42), stimulate adrenocorticotropic hormone secretion, or elevate heart rate and blood pressure (44). A further advantage in targeting IGFBPs is that it may be possible to identify nonpeptide small molecules that act as IGFBP ligand inhibitors, with the potential for good blood–brain barrier penetration and oral activity.

In summary, our data demonstrate that pharmacological elevation of "free" endogenous IGFs in the brain confers protection in a clinically relevant model of stroke. Because of the dramatic protection observed with this strategy, even when treatment is delayed for 1 h after occlusion of the artery, these data suggest that displacement of IGFs from IGFBPs in the brain is a potential treatment for stroke. Moreover, in view of the potent actions of IGFs on survival of neurons and glial cells as well as the widespread protective effects against a variety of brain insults, IGFBP ligand inhibitors may have broader utility for the treatment of various neurodegenerative disorders as well as traumatic brain and spinal cord injury.

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Insulin-Like Growth Factor-I (IGF-I) and Transforming Growth Factor-β1 Release IGF-Binding Protein-3 from Human Fibroblasts by Different Mechanisms*

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ABSTRACT

Human neonatal fibroblasts in monolayer culture secrete insulin-like growth factor-binding proteins (IGFBPs), which may modulate IGF action. To examine whether an increase in extracellular concentrations of IGFBPs in response to IGF-I is due to the release of cell-associated IGFBPs, we measured secreted and cell-associated IGFBP-3 immunologically in fibroblast monolayers treated with IGF-I and IGF analogs with altered affinities for the IGF receptors and IGFBPs. IGFBP-3 in medium conditioned by fibroblasts treated with IGF-I was significantly increased ($P < 0.05$) compared with that in medium from untreated cultures; concomitantly, cell-associated IGFBP-3 was significantly decreased ($P < 0.05$). [Ser^{24}]IGF-I (reduced affinity for IGF receptors) also increased secreted IGFBP-3 and decreased cell-associated IGFBP-3. In contrast, IGFBP-3 concentrations in medium conditioned by fibroblasts treated with B-chain IGF-I (reduced affinity for IGFBPs) were not significantly increased, and cell-associated IGFBP-3 was unchanged. Heparin, which releases proteins attached to cell surface proteoglycans, increased medium concentrations of IGFBP-3 and decreased IGFBP-3 binding to fibroblasts. An IGFBP of 29–31 kilodaltons (kDa) showed a pattern of regulation similar to that of IGFBP-3, while a third IGFBP, of 24 kDa, was decreased in IGF-I- and [Ser^{24}]IGF-I-conditioned medium and unchanged by B-chain IGF-I and heparin. Preincubation with transforming growth factor-β1 (TGFβ1), which stimulates fibroblast IGFBP-3 production, or human serum-derived IGFBP-3 did not increase cell-associated IGFBP-3. Analysis of total RNA isolated from fibroblasts revealed that IGFBP-3 mRNA was increased by TGFβ1, but not by IGF-I. These data suggest that IGFs and TGFβ1 release fibroblast IGFBPs by distinct mechanisms: IGFs by binding and subsequent release of cell-associated IGFBP-3 and 29- to 31-kDa IGFBP, and TGFβ1 by increased *de novo* synthesis of IGFBP-3. (*Endocrinology* **131**: 1703–1710, 1992)

THE INSULIN-like growth factors (IGF-I and IGF-II) bind with high affinity to specific IGF-binding proteins (IGFBPs), which are believed to play an important part in regulating the bioavailability and actions of the growth factors in their metabolic and anabolic roles. Six distinct classes of IGFBPs have now been identified and either purified or cloned from a wide variety of human and nonprimate sources (1). Of these, IGFBP-3 is the predominant IGFBP in the circulation, where it increases the half-life of IGF peptides (2) and may regulate IGF access to extravascular target tissues (3).

IGFBP-3 is also found in the conditioned medium of a wide variety of cell types *in vitro*, including vascular endothelial cells (4), neonatal and adult skin fibroblasts (5, 6), bone fibroblasts, chondrocytes, osteoblasts, and rhabdomyosarcoma cells (7), and human breast cancer cells (8, 9). Enhanced levels of IGFBP-3 and other IGFBPs in medium conditioned by cells in the presence of IGFs have been reported for human and bovine fibroblasts (10, 11), porcine, rat, and mouse myocyte cultures (12), and rat osteoblasts (13). Recent studies have shown that only IGF analogs that retain the ability to bind to IGFBPs are able to increase the concentration of IGFBP-3 in fibroblast culture medium (14, 15). In addition, the production of a 29- to 31-kilodalton (kDa) IGFBP in response to IGF stimulation of fibroblasts is apparently independent of interaction with an IGF receptor, as shown by the lack of stimulation by insulin at concentrations up to 1 μg/ml (10).

Exogenous serum-derived IGFBP-3 has been shown to both potentiate and inhibit the actions of IGFs *in vitro* (16–18) through mechanisms not yet fully understood. It has been suggested that inhibition of IGF action is through interaction with soluble IGFBP-3, while potentiation occurs via cell surface-associated IGFBP-3 (18). While some affinity labeling studies have shown cross-linked IGFBP-IGF complexes in solubilized cell extracts (18, 19), specific binding of IGFBP-3 to plasma membranes has not been demonstrated. In this study we have used an antibody to detect cell-associated IGFBP-3. Our results suggest that whereas the increase in medium IGFBP-3 in response to IGF-I results from the release of protein from cell surface or matrix proteoglycans, the stimulation of IGFBP-3 by transforming growth factor-β1 involves *de novo* synthesis.

Materials and Methods

Reagents

[Phe^{-1},Val^{1},Asn^{2},Gln^{3},His^{4},Ser^{8},His^{9},Glu^{12},Tyr^{15},Leu^{16}]IGF-I (B-chain IGF-I), [Gln^{3},Ala^{4},Tyr^{15},Leu^{16}]IGF-I ([QAYL]IGF-I), [Leu^{24}]IGF-I-(1–62), and [Ser^{24}]IGF-I were the generous gifts of Dr. M. Cascieri (Merck, Sharpe, and Dohme, Rahway, NJ). The specificities of these synthetic peptides for the IGF receptors and IGFBPs have been described previ-

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ously (14, 20–23). IGF-I, IGF-II, and IGFBP-3 were purified from Cohn fraction IV, as previously described (24, 25). The IGFBP-3 cDNA used in RNA analysis was generously provided by Dr. W. Wood (Genentech, Inc., South San Francisco, CA). Transforming growth factor-β1 (TGFβ1) from human platelets was purchased from British Biotech (Oxford, England), and heparin from porcine intestinal mucosa (grade 1; 178 USP units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal antiserum to IGFBP-3 (R7) was identical in specificity to antiserum R1–4, prepared as previously reported (26). When used in RIA, this antiserum is specific for primate IGFBP-3, and its reactivity with pure IGFBP-3 is unaffected by the presence of IGFs at concentrations up to 1 μg/ml (26). Normal rabbit serum was obtained from Endocrine Sciences (Tarzana, CA). Tracer for use in the IGFBP-3 RIA was prepared, as previously reported, by affinity labeling pure plasma IGFBP-3 with [^{125}I]IGF-I (26). IGF-I and IGF-II were radioiodinated using Na^{125}I and chloramine-T to a specific activity of 200 Ci/g and purified as previously reported (24, 27); [^{125}I]protein-A was prepared to a specific activity of approximately 100 Ci/g. Ham's F-12 medium and Dulbecco's Modified Minimal Essential Medium (DMEM), fetal calf serum, and glutamine were purchased from Cytosystems (North Ryde, New South Wales, Australia). Plasticware for tissue culture was purchased from Corning (Corning, NY) and Nunc (Roskilde, Denmark).

Cell cultures

Neonatal foreskin fibroblasts were derived from fibroblast outgrowth from circumcision tissue explants and maintained as monolayer cultures in Ham's F-12 medium supplemented with 20 mM HEPES, 2 mM glutamine, and 10% fetal calf serum (5). For stimulation experiments, confluent stock cultures were trypsinized and replated at a density of 8,000–10,000 cells/cm^2 in 24-place multiwell dishes in a serum-containing 4:1 mixture of DMEM-Ham's F-12 supplemented with HEPES and glutamine, as described above, and 0.06 g/liter penicillin, 0.1 g/liter streptomycin sulfate, and 0.5 g/liter BSA (DMEM:F-12). After 3 days, fibroblast monolayers were rinsed twice with serum-free DMEM:F-12 and incubated for a further 48 h in serum-free DMEM:F-12. Test substances (IGFs, heparin, *etc.*) were added in triplicate in 0.5 ml medium, and incubations were continued for 24 h. Conditioned media were collected and stored frozen until assayed for IGFBP-3, and cell monolayers were assayed for IGFBP-3 immunoreactivity as described below.

RIA for IGFBP-3 and antibody binding assay

Fibroblast-conditioned media (10–25 μl) were assayed for immunoreactive IGFBP-3, as described previously, using R7 antiserum at a 1:10,000 final dilution (5, 26).

To examine the possibility of specific association of IGFBP-3 with cells, we measured binding of R7, an antibody directed against IGFBP-3, to fibroblast monolayers. At the end of stimulation experiments, cell-conditioned media were removed, and the cell monolayers were incubated with 0.5 ml DMEM:F-12 containing R7 antibody at a final dilution of 1:1,000. After 16 h at 22 C, monolayers were rinsed twice with 1 ml serum-free DMEM:F-12 and incubated with 20,000 cpm [^{125}I]protein-A in 0.5 ml DMEM:F-12 for 2 h at 22 C. Fibroblasts were then washed a further two times with 1 ml DMEM:F-12, and cells were solubilized with 0.5 ml 5 g/liter sodium dodecyl sulfate (SDS). The resulting lysates were collected, and radioactivity was determined in a γ-counter. Nonspecific effects in the assay were determined by incubating duplicate experimental cultures with nonimmune rabbit serum instead of primary antibody, then processing as described above. The results of this assay are expressed as percent bound of the total added [^{125}I]protein-A.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and ligand blotting

Conditioned media were 4-fold concentrated by lyophilization and reconstitution in Laemmli sample buffer in preparation for electrophoretic analysis; typically, 80 μl from each of three triplicate wells were pooled and freeze-dried, then taken up in 60 μl sample buffer to give final concentrations of 0.0125 M Tris-HCl (pH 6.8), 30 g/liter SDS, 10% glycerol, and 0.5 g/liter bromphenol blue (28). Dissolved samples and calibration standards in the mol wt range 94–14 kDa (Pharmacia, Uppsala, Sweden) were placed in a boiling water bath for 5 min and allowed to cool before applying to resolving gels.

Prepared samples and standards were fractionated under nonreducing conditions on 10% SDS-polyacrylamide gels overnight at 100 V, then transferred electrophoretically to Hybond-C Extra supported nitrocellulose (Amersham, Aylesbury, Buckinghamshire, United Kingdom), using the procedure previously described (10). After transfer, IGFBPs were detected by ligand blotting with [^{125}I]IGF-II, basically according to the method of Hossenlopp *et al.* (29). Briefly, nitrocellulose sheets were incubated for 3 h at 37 C in Tris-buffered saline (TBS; 10 mM Tris and 150 mM NaCl, pH 7.4) containing 10 g/liter BSA, then overnight at 4 C in about 1 × 10^6 cpm [^{125}I]IGF-II in TBS containing 10 g/liter BSA and 0.05% Nonidet P-40. Blots were rinsed three times in cold TBS buffer, once in TBS buffer containing 0.05% Nonidet P-40, and three times in detergent-free TBS. Hyperfilm-MP autoradiographic film (Amersham) was exposed to dried blots for 1–3 days at −70 C, then developed.

Analysis of fibroblast IGFBP-3 mRNA

To analyze IGFBP-3 mRNA, fibroblasts were grown to confluence in tissue culture plates (10 cm diameter), then treated with IGF-I (50 ng/ml) or TGFβ1 (1 ng/ml), as described for stimulation experiments. A minimum of five plates of confluent cells was required for each RNA preparation. Total RNA was isolated from fibroblasts using a single step acid guanidinium thiocyanate-phenol chloroform extraction, according to the method of Chomczynski and Sacchi (30), and quantitated by absorbance at 260 nm. For Northern blots, 15 μg total RNA were denatured by heating to 65 C for 10 min in sample buffer containing final concentrations of 50% formamide, 6% formaldehyde, 1 × MOPS buffer [0.2 M MOPS (3-[*N*-morpholino]propanesulfonic acid), 5 mM sodium acetate, and 1 mM EDTA, pH 7.5] and 0.01% bromphenol blue. Immediately before loading, 2 μl ethidium bromide solution (1 mg/ml) were added to each tube. Samples were fractionated by electrophoresis through a 1% agarose gel containing 0.6 M formaldehyde, and the ethidium bromide-stained RNA was photographed under UV light. RNA was capillary-transferred to Hybond-N$^+$ nylon membrane (Amersham). Blots were hybridized with the [^{32}P]deoxy-CTP-labeled cDNA probe for human IGFBP-3 (31) overnight at 42 C in buffer [0.04 M sodium phosphate (pH 7.7), 4 mM EDTA, 0.72 M NaCl, 50% formamide, 1% SDS, 10% dextran sulfate, and 0.5 mg/ml sheared herring sperm DNA], then washed under high stringency conditions (as recommended by the manufacturer), dried, and autoradiographed. For slot blots, RNA was denatured by incubation for 15 min at 65 C in 50% formamide and 6% formaldehyde, then chilled on ice. Diluted samples containing 1–15 μg total RNA were applied to nylon membranes using a slot blot apparatus (Bio-Rad, Richmond, CA), hybridized with the labeled IGFBP-3 cDNA probe, then washed, dried, and autoradiographed, as described above.

Statistical analysis

Statistical analysis was carried out using the Statview II package for Apple Macintosh (Abacus Concepts, Inc., Berkeley, CA). Differences between groups of data were determined using Student's *t* test for two-group data sets, and analysis of variance and Fisher's protected least significant difference (PLSD) for multiple sets of data.

Results

The production of immunoreactive IGFBP-3 in response to stimulation by natural and synthetic IGFs was determined by incubating human fibroblasts with 50 ng/ml IGF-I or synthetic IGF-I analogs for 24 h, then assaying conditioned medium for IGFBP-3 by RIA. Figure 1 shows the increase in medium concentrations of IGFBP-3 in the presence of IGFs compared to that in fibroblasts incubated for the same 24-h period without added IGFs. Over 15 experiments, the addi-

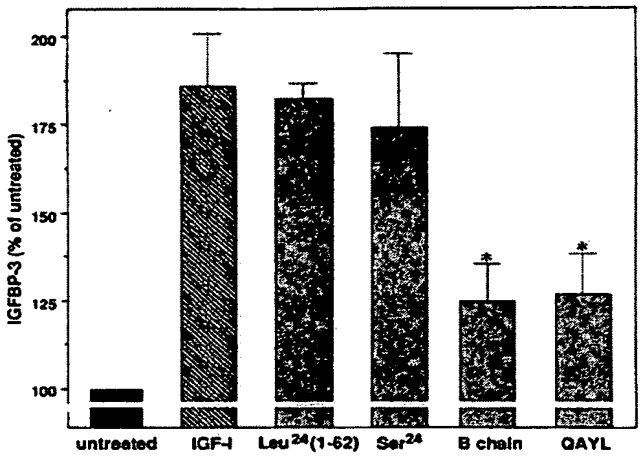

FIG. 1. IGFBP-3 in medium conditioned by fibroblasts treated with IGF-I and IGF-I analogs. Monolayers of human fibroblasts were incubated for 24 h without ('untreated') or with 50 ng/ml IGF-I or IGF-I analogs, as indicated. Conditioned media were collected and assayed for IGFBP-3 by RIA, as described in *Materials and Methods*. Results are expressed as percent stimulation (mean and SE) of IGFBP-3 in treated cultures compared with that in untreated cultures. *, $P < 0.05$ compared with IGF-I treatment (by analysis of variance and Fisher's PLSD).

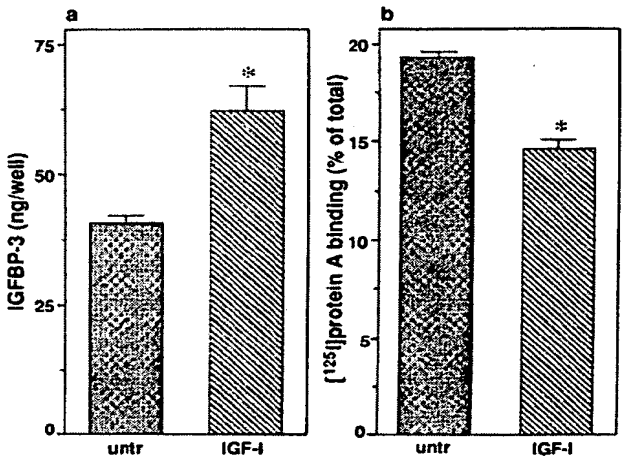

FIG. 2. Production of IGFBP-3 and binding of anti-IGFBP-3 antibody to human fibroblasts. Cultures of fibroblasts were incubated without (untr) or with 200 ng/ml IGF-I, as indicated, for 24 h. Media were removed for assay of IGFBP-3 and cell-associated IGFBP-3 by binding of anti-IGFBP-3 antiserum (R7), carried out as described in *Materials and Methods*. Bound antibody was detected by incubation with [^{125}I] protein-A, followed by solubilization of cells and γ-counting. Results are expressed as the radioactivity in the cell lysate, as a percentage of the total added [^{125}I]protein-A. *, $P < 0.05$ compared with untreated cultures, by two-tailed t test.

tion of IGF-I stimulated IGFBP-3 levels in conditioned medium by 87.4 ± 13.4% (mean ± SE), with a range of 30–350%. Similar increases in IGFBP-3 concentration were detected in medium samples from cells treated with both [Leu^{24}]IGF-I-(1–62) and [Ser^{24}]IGF-I, analogs that bind to IGFBPs with near-normal affinity for IGFBP-3 (23), but have reduced affinity for the IGF receptors (20, 22). These increases were not significantly different from the increase observed with IGF-I. In contrast, IGFBP-3 concentrations in medium conditioned in the presence of B-chain IGF-I and [QAYL]IGF-I, analogs that bind with normal affinity to the type 1 IGF receptor but only poorly to IGFBP-3, were not significantly increased compared to those in untreated samples ($P > 0.05$, by two-tailed t test) and were significantly lower than those observed with IGF-I and the IGFBP-3-binding IGF analogs ($P < 0.05$). These results confirmed that only IGFs that bind IGFBP-3 increase the secretion of IGFBP-3 from human fibroblasts.

To examine the possibility that the observed receptor-independent increase in IGFBP-3 is due to the release of cell-associated IGFBP-3, as has been suggested (18), we measured binding of a polyclonal antiserum (R7) to IGFBP-3 in untreated and IGF-I-treated fibroblast monolayers. The concentration of IGFBP-3 in medium conditioned by cells in the presence of 200 ng IGF-I was increased approximately 1.5-fold compared with that in untreated fibroblasts, as observed previously for 50 ng/ml IGF-I (Fig. 2). At the same time, binding of antibody to the fibroblast monolayers, expressed as the percent binding of [^{125}I]protein-A, was significantly decreased. Nonspecific tracer binding, determined by incubating treated and untreated monolayers with nonimmune rabbit serum before detection with [^{125}I]protein-A, was 1–2% and was not displaced by the addition of IGF-I (not shown).

To further examine the interaction between IGFs and cell-associated IGFBP-3, fibroblasts were incubated for 24 h with IGF-I, B-chain IGF-I, or [Ser^{24}]IGF-I in the concentration range 2–200 ng/ml before assay for antibody binding and medium IGFBP-3 concentrations (Fig. 3). When fibroblasts were incubated with IGF-I, IGFBP-3 levels in the medium were significantly increased at IGF-I concentrations greater than 10 ng/ml, with a concomitant decrease in cell-associated IGFBP-3 over the IGF concentration range examined. Similarly, an increase in the medium concentration of IGFBP-3 and a decrease in antibody binding were observed with fibroblasts incubated with [Ser^{24}]IGF-I. In contrast, incubation with B-chain IGF-I resulted in no significant increase in IGFBP-3 levels in the conditioned medium, and antibody binding to fibroblast monolayers was unchanged in the presence of this synthetic IGF-I analog. These results are consistent with the release of cell-associated IGFBP-3 by interaction with exogenous IGF-I. A time course of incubation of fibroblasts with IGF-I (50 ng/ml) indicated that significant displacement of IGFBP-3 occurred within 30 min of the addition of IGF-I, with half-maximal displacement achieved within approximately 2 h (not shown).

The carboxy-terminal regions of IGFBP-3 and other IGFBPs are rich in basic amino acids, and as such might interact with polyanions, such as sulfated glycosaminoglycans present on fibroblasts as proteoglycans in the cell membrane or extracellular matrix, or in peripheral association with the cell membrane. Such an interaction would be likely to be disrupted by heparin (32). To examine the possibility that the mechanism by which IGFBP-3 associates with fibroblasts is through interaction with a proteoglycan, cell-associated and medium IGFBP-3 were determined after cells were

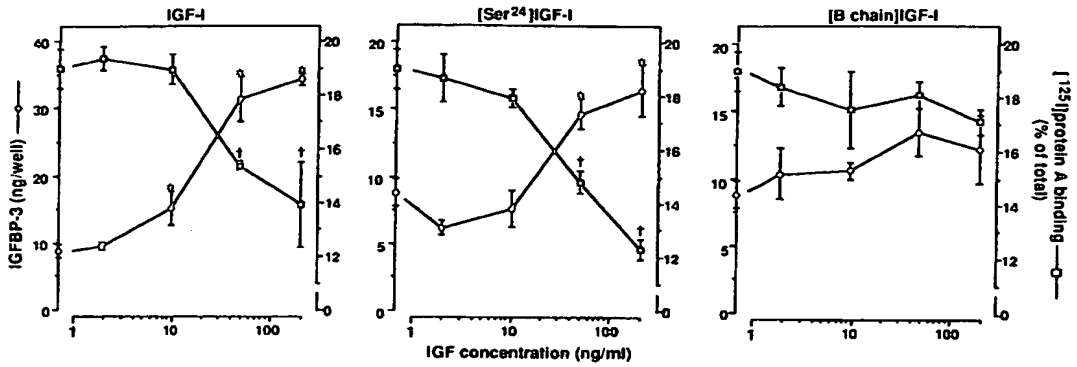

FIG. 3. IGF-I and IGF-I analog dose-response curves: medium IGFBP-3 and antibody binding to fibroblast monolayers. Fibroblast cultures were incubated with IGF-I (*left panel*), [Ser^{24}]IGF-I (*center panel*), or B-chain IGF-I (*right panel*) in the concentration range 2–200 ng/ml for 24 h. Conditioned media were assayed for IGFBP-3 (○), and antibody binding to fibroblast monolayers (□) was determined, as described in *Materials and Methods*. The mean and SE for triplicate wells from a single experiment are shown; similar results were obtained in three experiments. Significant differences shown ($P < 0.05$) were determined by analysis of variance and Fisher's PLSD: °, concentration of IGFBP-3 in medium significantly different from that in the absence of IGFs; †, antibody binding significantly different from that in the absence of IGFs.

treated with heparin over the concentration range of 1–100 μg/ml. As shown in Fig. 4, incubation with increasing concentrations of heparin caused a dose-dependent increase in IGFBP-3 levels in conditioned medium, with a half-maximal response at 3–4 μg/ml and a maximum response at 10 μg/ml. At concentrations greater than 30 μg/ml, an apparent decrease in IGFBP-3 levels in the medium was observed. Over the same concentration range, a significant decrease in R7 antibody binding to the fibroblast monolayers was apparent, with 30 μg/ml heparin causing maximal displacement of antibody. The release of IGFBP-3 by heparin suggests that the association of IGFBP-3 with fibroblasts *in vitro* occurs as a result of interaction with a proteoglycan in the membrane or matrix of the cell.

We have previously reported the IGF inducibility of a fibroblast IGFBP of 29–31 kDa by IGF-I and IGF-II (10). To establish whether the mechanism of induction of this IGFBP is similar to that observed for IGFBP-3, *i.e.* through the release of cell-associated IGFBP by binding to IGFs, medium conditioned by fibroblasts in the presence of increasing concentrations of IGF-I, [Ser^{24}]IGF-I, or B-chain IGF-I was fractionated by SDS-PAGE and ligand blotted with [^{125}I]IGF-II (Fig. 5a). The presence of 29- to 31-kDa IGFBP was evident in medium conditioned by fibroblasts treated with IGF-I and [Ser^{24}]IGF-I, increasing with concentrations of 50 ng/ml IGF and higher. This IGFBP species was not apparent in medium conditioned in the presence of B-chain IGF-I, suggesting that this protein, like IGFBP-3, may be cell associated and released by binding with IGFs. A 35-kDa IGFBP also appeared to be increased in response to IGF-I and [Ser^{24}]IGF-I in this experiment; however, an increase in this IGFBP was not consistently observed. Another IGFBP, of 24 kDa, was decreased by increasing concentrations of IGF-I and [Ser^{24}]IGF-I and remained unchanged in samples stimulated with B-chain IGF-I, while an IGFBP of 38 kDa was neither increased nor decreased by IGF treatment. Electrophoretic and ligand blot analysis of medium conditioned by fibroblasts treated with 3–300 μg/ml heparin (Fig. 5b) indicated a dose-dependent increase in the 29- to 31-kDa IGFBP, similar to that seen with IGF-I and [Ser^{24}]IGF-I; however, there was no change in the level of 24-kDa IGFBP over this range of heparin concentration. These findings indicate that the increase in secreted 29- to 31-kDa IGFBP, like that of IGFBP-3, occurs as a result of IGFBP interaction with IGFs rather than through an IGF-receptor mediated event, and suggest that a proteoglycan is involved in this association.

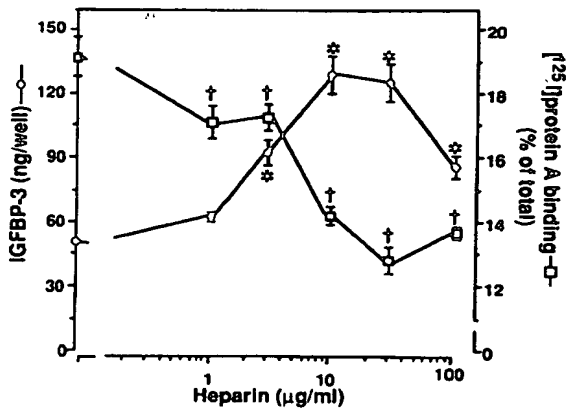

FIG. 4. Heparin dose-response curves: medium IGFBP-3 and antibody binding to fibroblast monolayers. Fibroblasts were incubated with heparin in the concentration range 1–100 μg for 24 h, then conditioned media were assayed by RIA for IGFBP-3 (○), and antibody binding to the monolayer was determined (□). Results are expressed as the mean and SE of triplicate determinations from a single representative experiment of a total of three experiments. ° and †, $P < 0.05$ compared with no heparin (by analysis of variance and Fisher's PLSD).

We were then interested in determining whether serum IGFBP-3, which is similar to fibroblast IGFBP-3 in size and IGF-binding characteristics (5), is able to associate with fibroblast monolayers. Cells were incubated for 24 h with pure serum IGFBP-3 at concentrations of 50, 200, and 1000 ng/ml, then the media were removed, and the cell monolayers were washed. Cell-associated IGFBP-3 was determined (Fig. 6). Over this concentration range, there was no increase in antibody binding to the fibroblast monolayers, suggesting

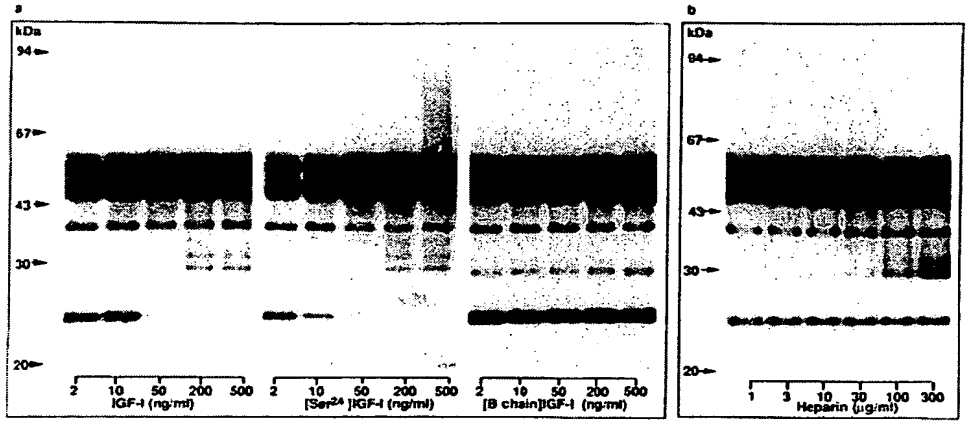

FIG. 5. [^{125}I]IGF-II ligand blot of IGFBPs in medium conditioned by IGF- and heparin-treated fibroblasts. Media conditioned for 24 h in the presence of 2–500 ng/ml IGF-I, [Ser^{24}]IGF-I, or B-chain IGF-I (a), or 1–300 μg/ml heparin (b),were prepared for SDS-PAGE, as described in *Materials and Methods*. Samples were fractionated on 10% gels, then transferred to nitrocellulose and probed overnight with [^{125}I]IGF-II. Autoradiography was performed for 3 days at −70 C. Migration distances of molecular mass markers, run in adjacent lanes of the gels, are indicated in kilodaltons on the *left*.

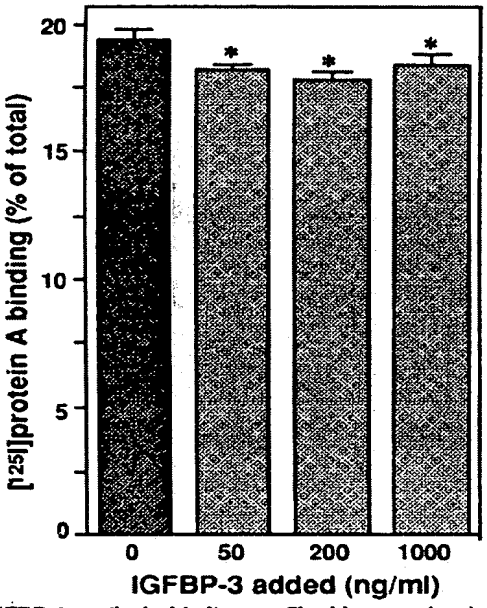

FIG. 6. IGFBP-3 antibody binding to fibroblasts preincubated with IGFBP-3. Human serum-derived IGFBP-3 (25) was diluted in medium and added to fibroblast cultures at the concentrations indicated for 24 h, then removed before determination of antibody binding to the fibroblast monolayer, as described in *Materials and Methods*. Results shown are the mean and SE of triplicate determinations from a single experiment; similar results were obtained in two experiments. *, $P < 0.05$ compared with no added IGFBP-3 (by analysis of variance and Fisher's PLSD).

that exogenous serum-derived IGFBP-3 did not associate with fibroblasts *in vitro*; in fact, a slight decrease in antibody binding to the cells was observed at all concentrations of added IGFBP-3 ($P < 0.05$). The reason for this is not known, but it is possible that added IGFBP-3 is not fully removed by the wash before incubation with antibody, thus competing with the fibroblast IGFBP for antibody-binding sites and causing an apparent decrease in cell binding.

TGFβ1 stimulates the production of IGFBP-3 by human neonatal fibroblasts (33). To determine whether this increase is due to the release of cell-associated IGFBP-3, as appears to be the case for IGF-stimulated increases in IGFBP-3 levels, IGFBP-3 antibody binding to fibroblast monolayers was examined after 24-h stimulation with 1 ng/ml TGFβ1 (Fig. 7). As previously observed, this level of TGFβ1 increased IGFBP-3 levels in fibroblast-conditioned medium approximately 2-fold over 24 h; over the same period of time, there was no significant change in antibody binding to the cell monolayers. These results suggest firstly, that stimulation of IGFBP-3 by TGFβ1 is not due to the release of cell-bound IGFBP-3, and secondly, that IGFBP-3 produced in response to stimulation by TGFβ1 does not remain associated with the fibroblasts.

Thus, it appeared that IGF-I and TGFβ1 increase extracellular concentrations of IGFBP-3 by different mechanisms: IGF-I by the release of cell-associated IGFBP-3, and TGFβ1 by increased *de novo* synthesis. To verify this, we used a radiolabeled IGFBP-3 cDNA probe to examine the relative abundance of IGFBP-3 mRNA in untreated fibroblasts and those treated with IGF-I or TGFβ1 (Fig. 8). A single hybridizing band of 2.5 kilobases was apparent in total RNA

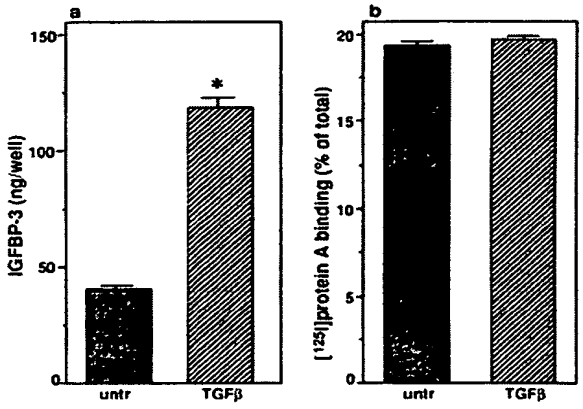

FIG. 7. Effect of TGFβ1 on antibody binding and medium IGFBP-3 concentrations. Cultures of fibroblasts were incubated without or with 1 ng/ml TGFβ1 for 24 h, as indicated. Assays for medium IGFBP-3 (a) and cell-associated IGFBP-3 (antibody binding; b) were carried out as described in *Materials and Methods*. *, $P < 0.05$ compared with untreated cultures (by two-tailed *t* test).

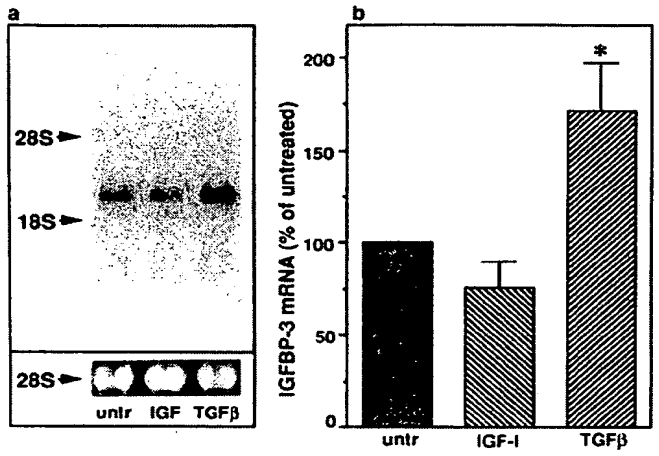

FIG. 8. Analysis of IGFBP-3 mRNA from IGF-I- and TGFβ1-treated fibroblasts. Total RNA was isolated from untreated fibroblasts and those treated with 50 ng/ml IGF-I or 1 ng/ml TGFβ1 for 2 days. a, Samples (15 μg) were run on 1% agarose formaldehyde gels, then hybridized with a ^{32}P-labeled IGFBP-3 cDNA probe, as described in *Materials and Methods*. The migration positions of 28S and 18S RNA are indicated on the *left*. The ethidium bromide-stained 28S RNA for these samples (to demonstrate equal loading of total RNA) is indicated at the *bottom* of the panel. b, Densitometric scans of Northern and slot blots of fibroblast RNA hybridized with the radiolabeled IGFBP-3 cDNA probe. Data were generated from three Northern blots and two slot blots of total RNA from three separate cultures of fibroblasts treated as indicated. *, $P < 0.05$ compared with untreated cultures (by two-tailed *t* test).

isolated from fibroblasts in the absence of growth factors (Fig. 8a). In the presence of IGF-I, there was no increase in the abundance of IGFBP-3 mRNA; however, IGFBP-3 message was increased in TGFβ1-treated fibroblasts. These results were confirmed by five densitometry scans of Northern and slot blots on RNA preparations from three different cultures of fibroblasts (Fig. 8b). A slight decrease in IGFBP-3 message was apparent in IGF-I-treated fibroblasts (not significant), while TGFβ1 caused a 1.5- to 2-fold increase in IGFBP-3 mRNA ($P < 0.05$).

Discussion

It has been suggested that some of the IGFBPs synthesized by fibroblasts *in vitro* remain associated with the cell (18, 19). This might explain an increase in the concentration of IGFBP-3 in fibroblast-conditioned medium (detected by SDS-PAGE and ligand blotting) in response to stimulation with IGF analogs with decreased affinity for IGF receptors but normal reactivity with IGFBPs (11, 14). In addition, affinity labeling of human fibroblast monolayers and subsequent SDS-PAGE analysis of solubilized cell extracts indicate the presence of IGF-IGFBP complexes ranging in size from 43–48 kDa (14, 19). None of these studies, however, has explicitly demonstrated interaction between the fibroblasts and IGFBP-3, either at the cell surface or in the extracellular matrix, or suggested a mechanism by which such an association may occur.

In this study we have shown directly that IGF-stimulated increases in the concentration of IGFBP-3 in fibroblast-conditioned medium occur concomitantly with displacement of cell-associated IGFBP-3 in the presence of IGFs. We also found that the addition of increasing concentrations of purified heparin to fibroblast monolayers resulted in a dose-dependent decrease in antibody binding and an increase in IGFBP-3 concentrations in the medium. Other studies have shown that a protein bound noncovalently with a cell-associated glycosaminoglycan can be displaced by analogs of that glycosaminoglycan; for example, lipoprotein lipase can be released from the endothelium by heparin (34). Thus, the association between the fibroblast and IGFBP-3 may be mediated by either integral membrane proteoglycans or glycosaminoglycans on the external surface of the cell or as a component of the extracellular matrix. The relative abundance of basic amino acids in the carboxy-terminal region of IGFBP-3 (31) suggests that it has the potential to bind with the polyanionic sulfated glycosaminoglycans known to be synthesized by and associated with human fibroblasts in culture (35). As IGFBP-3 lacks an arginine-glycine-aspartic acid (RGD) tripeptide sequence (31), which is a cell recognition site in many adhesive proteins (36), it is possible that the interaction with the cell is mediated through such proteoglycans, rather than through a specific receptor for IGFBP-3.

Human serum-derived IGFBP-3 did not bind to the fibroblast monolayer and, in fact, caused a slight displacement of fibroblast-derived IGFBP-3 from the monolayer. This was unexpected, since our earlier observation that incubation of fibroblasts with IGFBP-3 can potentiate IGF-I action has been interpreted by others as indicating a direct interaction between exogenous IGFBP-3 and the cell surface (37). Our result also contrasts with studies which report that increased nonreceptor binding of IGF tracers to the fibroblast cell surface coincides with the addition of serum-derived bovine IGFBP-3 (18, 38). There are a number of possible explanations for this apparent discrepancy. Firstly, bovine and human IGFBP-3 differ by nearly 20% (39) and may thus display different cell association characteristics. Secondly, there are detectable glycosylation differences between serum IGFBP-3 and that secreted by fibroblasts, which apparently do not affect its IGF-binding characteristics: the smaller component of the doublet predominates in fibroblast IGFBP-3 (10, 15), while the larger molecular mass component is the major species in serum IGFBP-3 (25). Thirdly, a lack of increased IGFBP-3 binding may reflect the fact that potential binding sites in the monolayer are already saturated with the endogenous fibroblast IGFBP-3. Further study is required to distinguish among these possibilities.

In contrast to the displacement of cell-bound IGFBP-3 by IGF-I, IGFBP-3 association with fibroblasts is unaltered by prior stimulation with TGFβ1. Our data, therefore, suggest 1) that the increase in IGFBP-3 as a result of stimulation with TGFβ1 is not due to the release of cell-associated IGFBP-3 (no decrease in antibody binding), and 2) that IGFBP-3 produced as a result of TGFβ1 stimulation does not remain associated with the cell in the same manner as IGFBP-3 produced by unstimulated cultures (no increase in antibody

binding). A lack of increase in cell-associated IGFBP-3 under conditions where production is highly stimulated by TGFβ1 may reflect saturation of available binding sites in the fibroblast monolayer, as suggested earlier for nonbinding of serum IGFBP-3. More importantly, our findings indicate that different mechanisms exist for the release of IGFBP-3 from fibroblasts by IGF-I and TGFβ1. Evidence for an alternate mechanism by which extracellular IGFBP-3 may be increased, that of increased *de novo* synthesis, has been provided in the demonstration of increased abundance of mRNA for IGFBP-3 in response to TGFβ1.

In addition to cell-associated IGFBP-3, we found that a 29- to 31-kDa IGFBP, previously identified as an IGF-inducible IGFBP (10), appears to associate with fibroblasts and is displaced from the cell by IGFs and heparin in much the same way as IGFBP-3. This supports our earlier finding that the 29- to 31-kDa IGFBP is not induced by insulin at very high concentrations, suggesting a mechanism of secretion independent of the IGF receptors (10). Conover (15) reported an increase in IGFBPs of 36 and 28 kDa in response to treatment of human dermal fibroblasts with IGF-I and [1–27,Gly_4,38–70]IGF-I, another IGF analog that binds normally to IGFBP but has reduced affinity for IGF receptors; as in our studies, non-IGFBP-binding analogs were without effect. We also observed a decrease in the medium concentration of an IGFBP of 24 kDa in samples from IGF-I- and [Ser^{24}]IGF-I-treated cells, but not [QAYL]-treated cells; a similar decrease in an IGFBP of this size has been reported for fetal and adult human fibroblasts (14, 15) and a squamous cell carcinoma line, SCL-1 (40). It is possible that the disappearance of this 24-kDa IGFBP is due to increased degradation of the IGFBP when complexed with IGFs.

To date, causal relationships between cell-associated or extracellular IGFBP-3 and modifications in the biological activity of IGFs have not been demonstrated unequivocally. A correlation between enhanced sensitivity and degree of response to IGF-I and increased nonreceptor binding of [^{125}I] IGF-I to bovine fibroblasts has led to the proposal of a dual role for IGFBP-3 in modulating IGF action at the cellular level, whereby extracellular IGFBP-3 inhibits IGF bioactivity by sequestration of the peptide, while surface-associated IGFBP-3 enhances IGF action (18). The existence of distinct mechanisms of release of IGFBP-3 from fibroblasts, as demontrated in our study, may reflect different roles for the cell-associated and extracellular forms of the BP in modulation of IGF activity.

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The C Region of Human Insulin-like Growth Factor (IGF) I Is Required for High Affinity Binding to the Type 1 IGF Receptor*

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We have produced and characterized the binding properties of three structural analogs of human insulin-like growth factor I (hIGF-I). These analogs are [1–62]hIGF-I, an analog lacking the carboxyl-terminal 8-amino acid D region of hIGF-I; [1–27,Gly^4,38–70]hIGF-I, an analog in which residues 28–37 of the C region of hIGF-I are replaced by a 4-reside glycine bridge; and [1–27,Gly^4,38–62]hIGF-I, an analog with the C region glycine replacement and a D region deletion. The removal of the D region of hIGF-I has little effect on binding to the type 1 and type 2 insulin-like growth factor (IGF) receptors. [1–62]hIGF-I has 2-fold higher affinity for the insulin receptor and 4-fold higher affinity for IGF serum-binding proteins. The replacement of the C region of hIGF-I with a four-glycine span results in a 30-fold loss of affinity for the type 1 IGF receptor. However this analog has near normal affinity for the type 2 IGF receptor, the insulin receptor, and IGF serum-binding proteins. Incorporating the C region glycine replacement and the D region deletion into one analog does not affect binding to either the type 2 receptor or to IGF serum-binding proteins. As predicted from the single deletion analogs [1–27,Gly^4,38–62]hIGF-I has reduced affinity for the type 1 IGF receptor (~40-fold) and increased affinity for the insulin receptor (5-fold). These data indicate that determinants in the C region of hIGF-I are involved in maintaining high affinity binding to the type 1 IGF receptor and that neither the C region nor the D region are required for high affinity binding to the type 2 IGF receptor or to IGF serum-binding proteins.

Human insulin-like growth factor I (hIGF-I)[1] is a 70-amino acid protein purified from human serum (1) that has been shown to promote cell growth and differentiation of various cell types (2). The biological effects of hIGF-I are mediated through its binding to one or more of three receptors. hIGF-I binds with highest affinity to the type 1 IGF receptor, a receptor structurally homologous to the insulin receptor (3, 4). hIGF-I also cross-reacts with lower affinity with the insulin receptor and with the type 2 IGF receptor. The type 2 IGF receptor is structurally and immunologically distinct from the type 1 IGF receptor and has no measurable affinity for insulin (3, 5). hIGF-I binds with high affinity to two classes of soluble binding proteins (6, 7). These proteins, which do not bind insulin, may modulate the biological activities of hIGF-I (8–10).

Human IGF-I shows a high degree of sequence homology with porcine and human insulin (1). Aligning residues 1–29 of hIGF-I with residues B2–B30 of insulin indicates that 45% of the residues are conserved. The positions of the cysteines (B7 and B19), both of which form interchain disulfide bonds in insulin, and the positions of all three glycines (B8, B20, and B23) which are also important for the insulin tertiary structure are identical in hIGF-I, hIGF-II, and insulin. hIGF-I also shows a high degree of sequence homology (52%) when the amino acids 42–62 are aligned with the A chain of insulin (A1–A21). Again the positions of the cysteines (A6, A7, A11, and A20) are conserved in hIGF-I, hIGF-II, and insulin. In hIGF-I the B (1–29) and A (42–60) regions are linked with a section of 12 amino acids (30–41) termed the C region or loop which is analogous to the C peptide of proinsulin. hIGF-II has an equivalent loop connecting the two regions but it contains only 8 residues. In addition, the carboxyl terminus of hIGF-I contains an 8-residue extension to the A region termed the D region. Human IGF-II contains a similar 6-residue extension.

The high degree of sequence homology between A and B regions of hIGF-I, hIGF-II, and insulin enabled Blundell and co-workers (11, 12) to build a three-dimensional model of hIGF-I (and hIGF-II) which is based closely in these regions on the x-ray coordinates of porcine insulin (13). To aid in the building of the C and D regions of hIGF-I, regions without structural data from x-ray crystallography, Blundell resorted to secondary structure prediction techniques (specifically the technique of Chou and Fasman (14)). The C region (30–41) was predicted to have two β-turns between residues 27–32. The remaining residues in this region are primarily hydrophilic and show no propensity for either helix or β-sheet formation, and the structure following the β-turns was built as an extended loop. In addition, the first of the two arginines in this region, Arg^{36}, was positioned to form an ion pair interaction with Asp^{45}. As a result of the β-turns and the Arg^{36}-Asp^{45} interaction, the side chain of Tyr^{31} is brought close to the Phe^{23}-Tyr^{24}-Phe^{25} region which is important in the binding of hIGF-I to the type I IGF receptor (15). Similar secondary structure prediction techniques applied to the D region suggested that the 8 residues have a propensity for anti-parallel β-sheet formation with the necessary β-turn occurring at Lys^{65} and Pro^{66}.

It is clear from such a model that both the C and D regions provide partial sheltering of the Phe^{23}-Tyr^{24}-Phe^{25} receptor binding region and by so doing may influence hIGF-I binding

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[1] The abbreviation used is: hIGF, human insulin-like growth factor I.

to the type I IGF or insulin receptors. One would predict from such a model that the ability of hIGF-I to bind to the insulin receptor should increase if mutants were prepared in which either the C or D region (or indeed both) were absent or reduced in size. In addition, the differences between hIGF-I and hIGF-II in both the C and D regions suggest that these regions may be important in determining the specificity of hIGF-I for the type I receptor. Further, the inability of insulin to bind to either the type II receptor or the IGF serum-binding proteins, suggests that either the C or D region, or both, may be important in these binding interactions.

In previous studies we have used site-directed mutagenesis of a synthetic gene encoding hIGF-I (15–19) and the subsequent expression of the mutant genes in yeast to identify residues in the A and B regions of hIGF-I important for maintaining binding to the types 1 and 2 IGF receptors, the insulin receptor, and IGF serum-binding proteins. In this report we examine the binding properties of structural analogs of hIGF-I lacking either the C region, the D region or both. Our data indicate that structural determinants in the C region are important in maintaining high affinity binding of hIGF-I to the type 1 IGF receptor. However, deletion of the D region does not dramatically alter binding of hIGF-I to the type 1 IGF receptor.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, MspI methylase and T4 DNA ligase were from New England Biolabs and used according to their recommendations. Crystalline bovine serum albumin came from Sigma. Bio-Rex 70 and Bio-Gel P-10 were from Bio-Rad. [^{125}I]Iodine was from Amersham Corp. and purified multiplication stimulating activity (rat IGF-II) was a gift from Dr. James Florini, Syracuse University.

DNA Manipulations—Standard DNA manipulations were carried out as described elsewhere (20). Oligodeoxynucleotides were synthesized by phosporamidite chemistry on an Applied Biosystems model 380A synthesizer and gel-purified as previously described (16). DNA sequences were determined on M13 templates or plasmid templates by the dideoxynucleotide chain termination method (21, 22). Plasmid strains were propagated in *Escherichia coli* strain DH5 following standard transformation procedures (23). Two rounds of transformations were carried out to avoid possible sequence heterogeneity in plasmids constructed with synthetic oligodeoxynucleotides.

Design and Construction of Mutant hIGF-I Genes—In order to examine the role of the D region of hIGF-I, the 3′ end of a synthetic gene for human IGF-I (16) was modified to encode [1–62]hIGF-I by replacing the 51-base pair *Xho*I/*Bam*HI fragment from pJY2 (17) with a 24-base pair synthetic fragment encoding amino acids 57–62 followed by a stop codon. This fragment was formed by the annealing of two oligonucleotides; 5′ TCGAAATGTACTGTGCTTGATAAG 3′ and 5′ GATCCTTATCAAGCACAGTACATT 3′. Digestion of pJY2 was limited to the *Bam*HI site at the 3′ end of the expression cassette by protecting the *Bam*HI site at the 5′ end using MspI methylase *in vitro*. After two rounds of transformation in *E. coli* DH5, a clone designated pJY62 was identified by *Bam*HI digestion and confirmed by DNA sequencing.

In order to examine the role of the C region of hIGF-I, a structural analog, [1–27,Gly^4,38–70]hIGF-I, was produced that replaced residues 28–37 of hIGF-I with a 4-residue glycine bridge. Examination of the x-ray structure of porcine insulin indicates that the minimum length peptide required to connect the B and A chains (about 10A) is 3 residues (13). The C region of hIGF-I, comprising 12 residues, easily spans the distance between the B and A regions in Blundell's model. Modeling of a selection of glycine oligomers designed to replace residues 28–37 in hIGF-I (10 residues in total in which the last 2 residues of the B region are replaced along with the first 8 residues of the C region) indicated that the glycine tetramer would be an adequate bridge.

Plasmid pJY161 encoding [1–27,Gly^4,38–70]hIGF-I was constructed by replacing the *Bst*EII/*Bgl*II of pJY2 (encoding amino acids 21–50) with a synthetic DNA fragment encoding amino acids 21–27,Gly^4,38–51. This fragment was formed by annealing and ligating four oligonucleotides 5′ GTGACCGCGGGTTCTACTTCAACAA GGGTGGTGGTGGT 3′, 5′ CGGAGCACCACCACCACCCTTGT TGAAGTAGAACCCGCG 3′, 5′ GCTCCGCAGACTGGTATCGT TGATGAATGCTGCTTCA 3′, and 5′ GATCTGAAGCAGCATTCA TCAACGATACCAGTCTG 3′ as previously described (16). After two rounds of transformation into *E. coli* DH5, a clone designated pJY161 was identified by *Xba*I digestion and confirmed by DNA sequencing.

Plasmid pJY223 encoding [1–27,Gly^4,38–62]hIGF-I was constructed by replacing the *Bgl*II/*Eco*RI fragment of pJY161 with the *Bgl*II/*Eco*RI fragment of pJY62. A clone designated pJY223 was identified by *Xba*I and *Xho*I digestion and confirmed by DNA sequencing.

The expression vector pα2 (17) contains a modified structural gene for the yeast mating factor MFα1 (24). *Bam*HI fragment of pJY62, pJY161, and pJY223 were cloned into the unique *Bam*HI site of pα2 to form pα2IGF62, pα2IGF161, and pα2IGF223, respectively; proper orientation for expression was determined by *Pst*I mapping using a *Pst*I site 213 base pairs upstream from the *Bam*HI site in pα2.

Expression and Purification of Mutant IGF-I Peptides—The mutant peptides were produced in *Saccharomyces cerevisiae* strain BJ1995, a gift of Dr. Elizabeth Jones, Carnegie Mellon University. The peptides were purified from the conditioned media in three steps as described previously (17). Briefly, medium was batch-treated with Bio-Rex 70 to absorb peptides which were then eluted with 1 M ammonium acetate, pH 8. Receptor-active material was pooled, applied to Bio-Gel P-10, and eluted in 1 N acetic acid. The receptor-active material was purified to homogeneity using C_{18} reverse-phase chromatography using a 15–50% acetonitrile gradient in 0.05% trifluoroacetic acid. Purity of the peptides was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 14% gels followed by electrophoretic transfer to nitrocellulose and gold staining as previously described (17).

Characterization of the Biological Activities of Mutant IGF-I Peptides—Receptor and binding protein assays were performed as previously described (17, 18). Briefly, type 1 IGF receptor affinity and insulin receptor affinity were determined by measuring the ability of peptides to inhibit the specific binding of ^{125}I-IGF-I (50–80 Ci/g, 0.25 nM) or ^{125}I-insulin (receptor grade, Du Pont-New England Nuclear, 0.6 mCi/ml), respectively, to human placental membranes. Type 2 receptor affinity was measured by determining the ability of peptides to inhibit the specific binding of ^{125}I-multiplication stimulating activity (30–50 Ci/g, 0.3 nM) to rat liver membranes. Serum-binding protein affinity was determined by measuring the ability of peptides to inhibit the specific binding of ^{125}I-IGF-I (0.3 nM) to acid-treated human serum. Nonspecific binding to the receptor and binding protein preparations was determined by adding excess unlabeled ligand and was ≤5% of total binding for all assays.

RESULTS

The strategy employed for the construction and expression of the mutant hIGF-I genes is described under "Experimental Procedures." In order to examine the role of the D region, a gene encoding [1–62]hIGF-I was constructed by inserting two translational termination codons following the DNA encoding the first 62 amino acids of hIGF-I. The DNA and protein sequence of the mutated region of the gene are shown in Fig. 1. Examining the role of the C region required that we delete as much as possible of this 12-residue region while maintaining the relationship between the A and B regions in the tertiary structure, thereby allowing proper disulfide bond

	Glu^{58}	Met^{59}	Tyr^{60}	Cys^{61}	Ala^{62}	*	*
A	GAA	ATG	TAC	TGT	GCT	TGA	TAA
	CTT	TAC	ATG	ACA	CGA	ACT	ATT

	Asn^{26}	Lys^{27}	Gly	Gly	Gly	Gly	Ala^{38}	Pro^{39}
B	AAC	AAG	GGT	GGT	GGT	GGT	GCT	CCG
	TTG	TTC	CCA	CCA	CCA	CCA	CGA	GGC

FIG. 1. DNA and protein sequences of the altered C and D regions of hIGF-I. *A*, [1–62]hIGF-I; *B*, glycine bridge of [1–27,Gly^4,38–70]hIGF-I.

formation. Based on the predicted three-dimensional structure of hIGF-I (11, 12) a 4-residue glycine bridge was included between residues Lys^{37} and Ala^{38} replacing residues 28–37. The DNA and protein sequence of this connecting region are shown in Fig. 1. A gene encoding [1–27,Gly^4,38–62]hIGF-I, a 56-amino acid structural analog lacking both the C and D regions was constructed by combining the appropriate DNA segments from the above genes as described under "Experimental Procedures."

The expression vectors were introduced into a protease-deficient strain of yeast, *S. cerevisiae* BJ1995, the yeast was grown to saturation in a 1-liter culture, and the peptides were purified from the conditioned media in three chromatographic steps. The purity of the peptides was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (17) (data not shown). The final yields of peptide from 1 liter of conditioned medium were 212 μg for [1–62]hIGF-I, 65 μg for [1–27,Gly^4,38–70]hIGF-I, and 700 μg for [1–27,Gly^4,38–62]hIGF-I.

[1–62]hIGF-I has normal affinity for the types 1 and 2 IGF receptors (Fig. 2, *A* and *D*). It has 2- and 4-fold higher relative affinity for the insulin receptor and human serum-binding proteins, respectively (Fig. 2, *B* and *C*). In contrast, [1–27,Gly^4,38–70]hIGF-I has 30-fold lower relative affinity than hIGF-I for the type 1 IGF receptor (Fig. 2*A*). This peptide has normal affinity for the type 2 IGF receptor and the insulin receptor (Fig. 2, *B* and *D*) and has 3-fold higher relative affinity for serum-binding proteins (Fig. 2*C*). The mutant in which these deletions are combined, [1–27,Gly^4,38–62]hIGF-I, also has drastically reduced relative affinity for the type 1 IGF receptor (Fig. 2*A*), 5- and 4-fold increased relative affinity for the insulin receptor and serum binding proteins, respectively (*B* and *C*) and normal affinity for the type 2 IGF receptor (*D*). Table I summarizes the relative affinities of these proteins for the receptors and binding proteins. These peptides are all full agonists at the type 1 IGF receptor as determined by their ability to stimulate the incorporation of [^{3}H]thymidine incorporation into DNA in the rat clonal vascular smooth muscle cell line, A10 (data not shown).

The biphasic slopes of the titration curves showing inhibition of ^{125}I-IGF I binding to acid-treated human serum-binding protein for [1–27,Gly^4,38–70]hIGF-I and [1–27,Gly^4,38–62]hIGF-I are different than the slope of the titration curve for hIGF-I and [1–62]hIGF-I (Fig. 2*C*). The bivalent cross-linking reagent disuccinimidyl suberate was used to covalently label the binding proteins present in acid-treated human serum with ^{125}I-IGF I. A major band of 42,000 and a minor band of 25,000 are specifically labeled in acid-treated serum. Labeling of these proteins is inhibited by [1–27,Gly^4,38–70] hIGF-I and [1–27,Gly^4,38–62]hIGF-I in a dose-responsive manner similar to that of hIGF-I (data not shown) to all species in the acid-treated serum. Explanation of the nature of the biphasic binding curve must await studies with purified binding proteins.

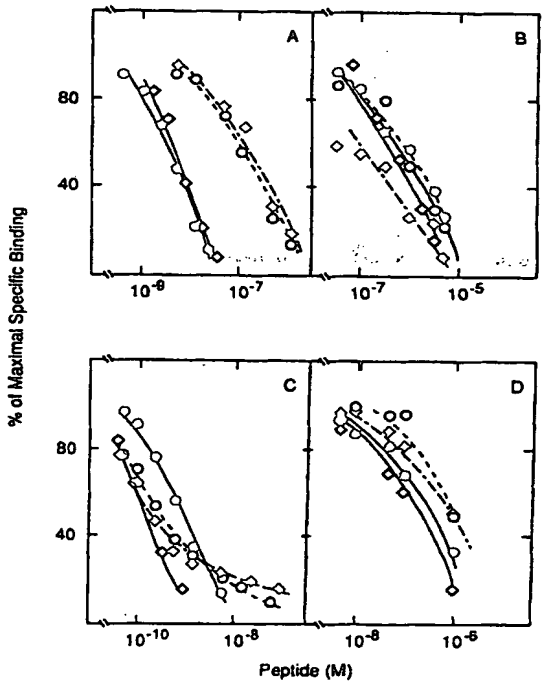

FIG. 2. **Inhibition of ligand binding to human placental type 1 IGF receptors (*A*), human placental insulin receptors (*B*), acid-stable human serum binding proteins (*C*), and rat liver type 2 IGF receptors (*D*) by hIGF-I (○), [1–62]hIGF-I (◇), [1–27,Gly^4,38–70]hIGF-I (○), and [1–27,Gly^4,38–62]hIGF-I (◇).** Data are expressed as the percent of maximal specific binding of ligand determined in the absence of added peptide. Each point represents the mean for two determinations.

DISCUSSION

In this study we examined the roles of the C and D regions in determining the binding properties of human IGF-I. These regions comprise two of the four major structural domains of hIGF-I and represent the regions not found in insulin. In the predicted three-dimensional structure of hIGF-I (Fig. 3*B*), these two regions appear to be closely associated with the aromatic residues Phe^{23}-Tyr^{24}-Phe^{25}, a region homologous to the receptor binding region of insulin, Phe^{B24}-Phe^{B25}-Tyr^{B26}. We have previously shown that these aromatic residues are important in type 1 IGF receptor binding (15).

In order to examine the role of the C region of hIGF-I, a structural analog was produced that replaced residues 28–37 of hIGF-I with a 4-residue glycine bridge. The resulting mutant peptide, [1–27,Gly^4,38–70]hIGF-I displayed a 30-fold reduction in relative affinity for the type 1 IGF receptor (Fig. 2*A*). These data are consistent with results of Tseng *et al.* (25) who demonstrated that a chemically synthesized two-chain molecule containing just the A and B domains of the IGF-I has only 10–15% of the potency of native IGF-I in binding to the type IGF receptor. Remarkably, binding of [1–27,Gly^4,38–70]hIGF-I to the insulin receptor, the type 2 IGF receptor, and to IGF-binding proteins appeared unaffected. These data indicate that the glycine bridge allowed proper formation of the overall tertiary structure of this analog. These data also support previous work in our laboratories which indicate that hIGF-I has separate binding domains for its receptors and binding proteins (15, 18, 19).

The absence of the D region of hIGF-I in [1–62]IGF-I has little effect on type 1 or 2 IGF receptor binding (Fig. 2, *A* and *C*). However, this analog does have 2-fold higher relative affinity for the insulin receptor (Fig. 2*B*). These data are in agreement with those obtained by King *et al.* (26) using a synthetic two-chain insulin-like peptide with a 29-residue A chain. The addition of the D region of hIGF-I to insulin resulted in a molecule with reduced binding to the insulin receptor (4-fold) and reduced activity in the glucose oxidation bioassay (5-fold). These data support the prediction of the computer-generated hIGF-I structure that the D region may sterically hinder hIGF-I binding to the insulin receptor (11).

The absence of the C region, the D region, or the absence

TABLE I

Effects of modifications in the C and D regions of hIGF-I on binding to the human placental type 1 IGF receptor, the rat liver type 2 IGF receptor, the human placental insulin receptor (IR) and human serum binding proteins (hBP).

Peptide	IC_{50}[a]			
	Type 1	Type 2	IR	hBP
	nM	*μM*	*μM*	*nM*
hIGF-I	4.9 ± 2	0.4 ± 0.3	1.4 ± 0.6	0.8 ± 0.4
[1–62]hIGF-I	6.4 ± 1.3	0.4 ± 0.1	0.7 ± 0.3	0.17 ± 0.01
[1–27,Gly[4],38–70]hIGF-I	145 ± 21	0.9 ± 0.2	0.8 ± 1	0.28 ± 0.04
[1–27,Gly[4],38–62]hIGF-I	205 ± 66	1.0 ± 0.1	0.3 ± 0.1	0.20 ± 0.05

[a] Mean ± S.D., $n = 2$ for mutants, $n = 6$ for hIGF-I.

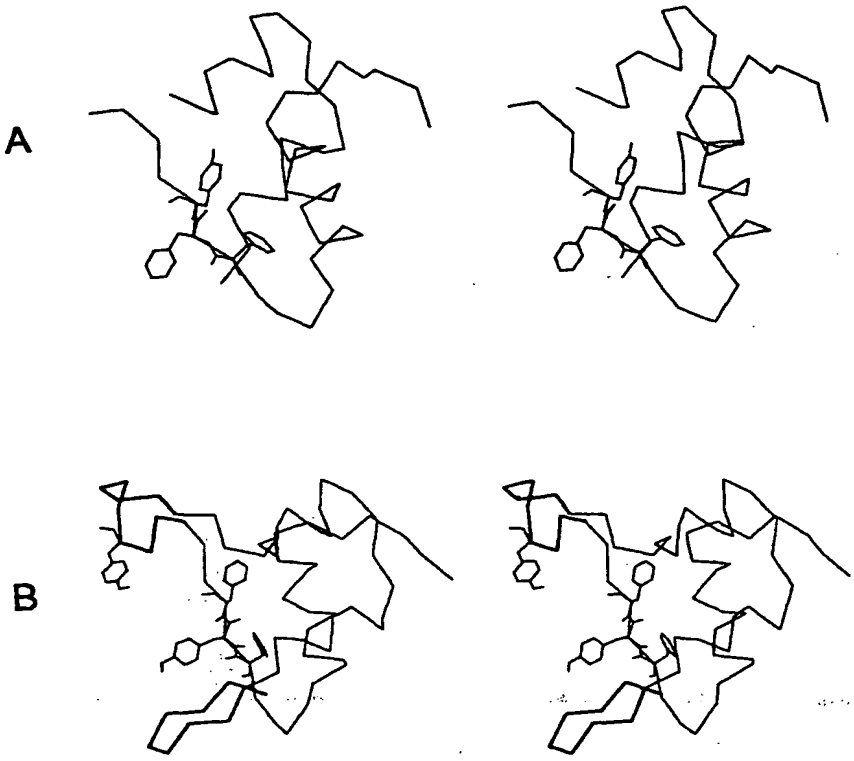

FIG. 3. **Stereo comparison of the crystal structure of porcine insulin (13) (*A*) with the proposed structural model of hIGF-I (11, 12) (*B*).** In the insulin structure, residues Phe[24],Phe[25] and Tyr[26] of the B-chain are shown. In the hIGF-I structure residues Phe[23],Tyr[24],Phe[25], and Tyr[31] are shown. The *C* and *D* regions of hIGF-I are *highlighted.*

of both regions did not reduce the affinity of the respective analogs to IGF binding proteins or to the type 2 IGF receptor (Fig. 2, *C* and *D*) indicating that these regions are not required to maintain high affinity binding to these proteins. These data complement previous work showing that the addition of the six-amino acid D region of rat IGF-II had no effect on the binding of insulin on the A insulin-B IGF-I hybrid to acid-stable rat serum-binding proteins (27).

We have previously shown that substituting residues 1–16 of hIGF-I with the corresponding residues in the B-chain of insulin produces a dramatic loss in affinity for the soluble IGF-binding proteins with no change in affinity for the type 1 IGF receptor (18, 28, 29). The simpler substitution of Gln[15]-Phe[16] of hIGF-I with the corresponding Tyr[B16]-Leu[B17] sequence in insulin results in a 5–10-fold increase in affinity for the insulin receptor with no change in affinity for the hIGF-I receptor (18). Similarly, substituting residues 42–58 in the A region of hIGF-I with the corresponding residues in the A-chain of insulin results in a 25-fold loss in relative affinity for the type 2 IGF receptor with no change in affinity for the type 1 IGF receptor (19). These data and the data in the present report strongly suggest that these two regions and the D region are not involved in the high affinity binding of hIGF-I to the type 1 IGF receptor, although residues 15 and 16 in the B region are involved in binding to the highly homologous insulin receptor.

We have shown that the aromatic residue at position 24 of hIGF-I (15) and residues contained within the 28–37 deletion sequence are important for maintaining type 1 IGF receptor binding. It is likely that the poor affinity of insulin for the type 1 IGF receptor is due to its lack of these determinants in the C region. Recent studies by Maly and Luthi (30) demonstrate that Tyr[31] is protected from iodination when bound to the type I IGF receptor, suggesting that this residue of the C region is involved in receptor binding. Preliminary data from our laboratories indicate that the substitution of Ala for Tyr[31] in hIGF-I results in a 10-fold loss of affinity for the type I receptor (31). More selective substitutions of residues in the C region will be required to further define amino acids important for receptor binding.

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Mechanisms of Sertoli Cell Insulin-Like Growth Factor (IGF)-Binding Protein-3 Regulation by IGF-I and Adenosine 3′,5′- Monophosphate*

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ABSTRACT

FSH, which stimulates cAMP in the Sertoli cell, markedly lowers the concentration of insulin-like growth factor-binding protein-3 (IGFBP-3) in Sertoli cell-conditioned medium; in contrast, insulin-like growth factor-I (IGF-I) increases BP-3 expression. In this study, the mechanisms controlling the contrasting effects of cAMP and IGF-I were investigated. The abundance of BP-3 mRNA was dramatically lowered by $(Bu)_2$cAMP, but was unaffected by IGF-I. Analyzed by ligand blot of conditioned medium, coincubation of $(Bu)_2$cAMP and IGF-I largely eliminated the increase observed with IGF-I alone. Based on the following evidence, the effect of IGF-I appeared to be solely related to the capacity of IGF-I to interact directly with BP-3. 1) Insulin at micromolar concentrations failed to increase BP-3 abundance despite documentation by affinity cross-linking that insulin displaced [^{125}I]IGF-I from the IGF-I receptor. 2) A synthetic IGF-I analog, [Leu^{24},1–62]IGF-I, which has reduced binding affinity for rat IGF-I receptor but displays high affinity for rat Sertoli cell-conditioned medium BPs, increased BP-3 abundance. 3) A synthetic IGF-I analog, B-chain mutant, which has reduced affinity for rat Sertoli cell BPs but displays normal affinity for the rat IGF-I receptor, failed to increase BP-3 abundance. 4) Human recombinant glycosylated [^{125}I]BP-3 when added to cultured Sertoli cells was preserved in the medium when IGF-I or analogs with BP-3 affinity were present. 5) IGF-I, in dose-responsive manner, both retarded the disappearance from the medium of exogenously added human recombinant nonglycosylated BP-3 and decreased the amount of membrane-associated BP-3. These results indicate that whereas cAMP lowers BP-3 abundance in medium, most likely by markedly decreasing synthesis, IGF-I increases BP-3 accumulation by retarding its clearance by the Sertoli cell. (*Endocrinology* **131**: 2733–2741, 1992)

THE INSULIN -like growth factors (IGFs) are thought to be important regulators of gonadotropin action in the testis (1–5). However, the biological activity of testicular IGF is likely to depend on the local concentration of specific, high affinity binding proteins (BPs) (6–13). We recently demonstrated that BP-3, one of six known IGFBPs (14), is the predominant IGFBP produced by rat Sertoli cells in culture (8). We also observed that FSH, which activates adenylate cyclase and increases cAMP (15), markedly lowers the concentrations of BP-3 in Sertoli cell-conditioned medium (8). Similarly, other agents that increase cAMP, such as isoproterenol and cholera toxin, and/or cAMP agonists, such as $(Bu)_2$cAMP, have similar inhibitory effects (8). As BP-3 has been shown to be an inhibitor of IGF activity on the cultured rat granulosa cell (16), it appears that the effect of FSH on BP-3 expression is likely to be integrated into the physiological function of FSH. In this paradigm, because FSH stimulates IGF-I production and IGF-I enhances FSH-stimulated steroidogenesis (17, 18), FSH may serve to amplify the actions of IGF by the concurrent increase in IGF-I and decline in BP-3.

However, in contrast to the effect of cAMP on BP-3, we previously demonstrated that IGF-I, as it does in other cultured cells, strikingly increases BP-3 concentrations in Sertoli cell-conditioned medium (8). This increase in BP-3 in response to IGF-I seems at cross-purposes with the FSH actions, particularly if the primary function of BP-3 is to inhibit the biological activity of IGF. To better understand the apparent conflicting effects of IGF-I and cAMP on BP-3 expression, we examined the mechanisms by which cAMP and IGF-I influence the abundance of BP-3 in Sertoli cell-conditioned medium. cAMP appears to reduce BP-3 concentrations by decreasing the concentrations of BP-3 mRNA. In contrast, the IGF-stimulated increase in BP-3 is independent of IGF-receptor interaction or increased synthesis resulting instead from direct interaction of IGF with secreted BP-3.

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Materials and Methods

Reagents

BP-3 cDNA was synthesized by the polymerase chain reaction, using a cDNA template derived from rat Sertoli cell poly(A)$^+$ RNA, as previously described (8). Androgen-binding protein cDNA was a 1.4-kilobase insert in Sp65 provided by David R. Joseph, Laboratories for Reproductive Biology, University of North Carolina (Chapel Hill, NC) (19). The IGF analogs, [Q^3,A^4,Y^{15},L^{16}]IGF-I (20, 21), [Leu^{24},1–62]-IGF-I (22), and B-chain mutant, were kindly provided by Margaret A. Cascieri, Merck, Sharp, and Dohme Research Laboratories (Rahway, NJ). When tested in human systems, [Q^3,A^4,Y^{15},L^{16}]IGF-I and B-chain mutant bind normally to the IGF-I receptor, but have markedly reduced affinity for BP-3; [Leu^{24},1–62]IGF-I has markedly reduced IGF-I receptor affinity, with normal binding to BP-3. IGF-I (Thr^{59}-IGF-I) was obtained from Amgen Biological, Inc. (Thousand Oaks, CA), or Intergen, Inc. (Purchase, NY); IGF-II was obtained from Amgen. *E. coli*-derived nonglycosylated human BP-3 (rBP-3), Chinese hamster ovary cell-derived glycosylated BP-

3 (rgBP-3), and antiserum to human *E. coli*-derived BP-3 were generous gifts from Andreas Sommer and Christopher A. Maack, BioGrowth, Inc. (Richmond, CA) (23). The antiserum to human BP-3, also provided by BioGrowth, is an unfractionated antiserum raised in rabbits to *E. coli*-derived BP-3; it is specific for human BP-3 and does not cross-react with rat BP-3. At a 1:5000 dilution, 10 ng *E. coli* BP-3 are easily detected by immunoblot analysis. Penicillin and streptomycin were obtained from Whittaker M. A. Bioproducts (Walkersville, MD), and gentamicin from Gibco (Grand Island, NY). $(Bu)_2$cAMP was obtained from Sigma (St. Louis, MO). Pure porcine insulin was obtained from Eli Lilly Co. (Indianapolis, IN).

Primary cell culture

Sertoli cell-enriched cultures were prepared from testes of 15-day-old Sprague-Dawley rats (Harlan, Indianapolis, IN) by standard techniques, as previously described (24). Cells were plated into 60-mm dishes in Eagle's Minimum Essential Medium supplemented with 1 mM pyruvate, 4 mM glutamine, nonessential amino acids, 250 U/ml penicillin, 250 U/ml streptomycin, 12.0 μg/ml gentamicin, and 5% fetal bovine serum (Gibco). After 24 h, confluent cell monolayers were washed once, and fresh medium without serum was added. Medium was changed on days 3 and 5 before initiating experiments. For Sertoli cell culture experiments, growth factors were added as 100-fold aliquots in Hanks' Balanced Salt Solution containing 1 mg/ml BSA (98–99% pure; fatty acid free; Sigma). Each treatment condition was performed in triplicate 60-mm dishes, with 3.0 ml/dish. The medium for each triplicate condition was pooled, 2-ml aliquots were concentrated to 50 μl, and 25 μl were used for ligand blotting. For experiments involving exogenously added recombinant BP-3, BSA (1 mg/ml) was also added to the Eagle's Minimum Essential Medium to further decrease nonspecific binding. In experiments involving recombinant BP-3, duplicate 35-mm dishes were used, with 2.0 ml/dish. The medium from each duplicate condition was pooled, and an 80-μl aliquot was used without prior concentration.

Because of the proclivity of BP-3 to nonspecifically bind to container surfaces and the possibility that the presence of IGF-I might alter the recovery of BP-3 during the Centricon concentration step, the effect of IGF-I on BP-3 abundance was directly compared in concentrated *vs.* unconcentrated samples. There was minimal difference in the percent increase between the two conditions (data not shown). Finally, to address whether the treatment conditions significantly changed the protein content of the cells, protein assays were performed on triplicate dishes after 48 h of treatment with either IGF-I (100 ng/ml) or FSH (100 ng/ml) or in control dishes without hormone. As expected, because the cells were plated at confluence and as Sertoli cells cultured from day 15 animals only minimally divided in culture, there were minimal differences in protein content (data not shown).

Ligand and Western blotting

Radioligand blotting with [^{125}I]IGF-I was performed by the method of Hossenlopp *et al.* (25). In most of the experiments, conditioned medium samples (2 ml) were concentrated in 30,000 mol wt cut-off Centricons (Amicon Corp., Danvers, MA) before electrophoresis. In experiments involving recombinant BP-3, 80 μl sample medium were directly electrophoresed. Samples were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. The proteins were transferred to 0.2-μm pore size nitrocellulose by electroblotting using a model TE50X Transphor Powerlid apparatus (Hoefer Scientific Instruments, San Francisco, CA). BPs were identified by incubation with [^{125}I]IGF-I (500,000 cpm/blot) and subjected to autoradiography.

Detection of rBP-3 associated with Sertoli cell membranes was determined by washing cells twice with 1.0 ml Minimum Essential Medium-0.1% BSA, followed by the addition of PBS. The cells were scraped off the dish, transferred to a microfuge tube, and briefly centrifuged to pellet the cells. The cell pellet was resuspended in 0.25 M sucrose, 10 mM Tris, and 1.0 mM EDTA, pH 7.4, at 4 C and disrupted with a ground glass homogenizer. The disrupted cells were centrifuged at 200 × *g* for 10 min to pellet nuclei, and the supernatant was centrifuged at 12,000 rpm in a microfuge at 4 C for 20 min. After aspirating the supernatant, the pellet was resuspended in 50 μl 0.01 M sodium phosphate buffer, pH 7.0, and stored at −20 C for electrophoresis.

For immunoblot analysis, blots previously incubated with [^{125}I]IGF-I were incubated with antihuman BP-3 antiserum at a final concentration of 1:5000 overnight and processed by standard methods using donkey biotinylated antirabbit immunoglobulin G second antibody (Chemicon, Temecula, CA) and Vectastain ABC-AP kit (Vector Laboratories, Inc., Burlingame, CA).

RNA isolation and analysis

Poly(A)$^+$ mRNA was isolated from cultured Sertoli cells by the method of Chirgwin *et al.* (26), as modified by Lund *et al.* (27). RNAs were fractionated by electrophoresis through 1% agarose-6% formaldehyde gels and transferred to BioTrans nylon membranes (ICN, Irvine, CA). The integrity of the RNA was validated by electrophoretic separation and staining of blotted membrane with methylene blue. BP-3 and androgen-binding protein RNA expression were analyzed by hybridization with ^{32}P-labeled cDNA probes (28) with specific activities of $1–3 \times 10^9$ cpm/μg.

Affinity labeling and displacement studies with Sertoli cell and B104 membranes

Crude membrane preparations were prepared from cultured Sertoli cells similar to those used for the BP-3 regulation experiments and from binding protein-negative rat neuroblastoma cells (B104) (29). Affinity labeling studies with Sertoli cell or B104 membranes were performed as described previously (30). Briefly, 500 μg plasma membrane were incubated with 500,000 cpm [^{125}I]IGF-I in the absence or presence of unlabeled Thr^{59}-IGF-I, IGF-II, Leu^{24}, B-chain mutant, or insulin (in a final volume of 0.5 ml 0.1 M phosphate buffer, pH 7.4) for 16 h at 4 C. Sixty microliters of 1.36 mM disuccinimidyl suberate (freshly prepared in dimethylsulfoxide) were then added and 30 min later, the affinity labeling reaction was quenched with 60 μl 1 M $(NH_4)_2HPO_4$ and 1 ml cold phosphate buffer. The membrane was pelleted by centrifugation at 12,000 rpm for 10 min and washed twice with 1 ml cold phosphate buffer. It was then fractioned in a 5%/7% discontinuous SDS-PAGE for autoradiography.

For displacement studies with B104 membranes, 50 μg of membranes were incubated (in triplicate) with 40,000 cpm [^{125}I]IGF-I in 50 mM Tris-HCl, pH 7.4, containing 0.1% BSA in the presence of graded concentrations of unlabeled IGF-I, Leu^{24}, or B-chain mutant. After incubation overnight at 4 C, the mixtures were centrifuged (Beckman microfuge, Palo Alto, CA), and membrane-bound radioactivity in the pellets was measured with a γ-counter. Specific binding was defined as the percentage of total counts per min bound minus the percentage bound in the presence of an excess of unlabeled IGF-I.

IGFBP-binding capacity assay

Charcoal assay (31) was performed by a previously described method to determine the relative affinity of BP in Sertoli cell-conditioned medium for IGF-I, Leu^{24}, and B-chain mutant. Specifically, 60 μl Sertoli cell-conditioned medium were incubated at 4 C overnight with 20,000 cpm [^{125}I]IGF-I in the presence of graded concentrations of IGF-I, Leu^{24}, or B-chain mutant. The samples were incubated with charcoal solution, as previously described (31), and unbound radioactivity was counted in the supernatant after centrifugation. The percent specific binding was equal to (sample counts − nonspecific binding counts)/total counts.

Iodination of BP-3

Iodination was performed using the standard chloramine-T method (30). Briefly, 5.2 μg human glycosylated BP-3 in 10 μl 20 mM potassium phosphate and 0.5 M NaCl, pH 7.0, were iodinated with 1 μCi (100 mCi/ml) ^{125}I by the addition of 8 μg chloramine-T in 20 μl 50 mM sodium phosphate, pH 7.2. After 2 min at room temperature, the reaction mixture was quenched with 8 μg sodium metabisulfite in 10 μl 50 mM sodium phosphate buffer, pH 7.2. Free ^{125}I was removed by chromatog-

raphy on a Sephadex G-75 column. Fractions with the maximal radioactivity were tested for integrity by RIA, using an antiserum to human BP-3 provided by BioGrowth, Inc., and by visualizing the iodinated species by electrophoresis on 12% SDS-PAGE.

Results

Effects of $(Bu)_2$cAMP and IGF-I on BP-3 mRNA abundance

To understand the opposite actions of cAMP and IGF-I on BP-3 abundance, we initially examined the effects on mRNA abundance. Figure 1 shows the result of blot hybridization of Sertoli cell poly(A)$^+$ mRNA derived from cells treated for 48 h with $(Bu)_2$cAMP, IGF-I, or control medium. In Fig. 1A, RNA was hybridized with rat BP-3 cDNA. There was little difference between control and IGF-I-treated samples, whereas the $(Bu)_2$cAMP-treated cells had undetectable levels. The blot was stripped and rehybridized with androgen-binding protein cDNA (Fig. 1B). In marked contrast to the changes in BP-3 mRNA after $(Bu)_2$cAMP treatment, androgen-binding protein mRNA was increased, as previously reported (19).

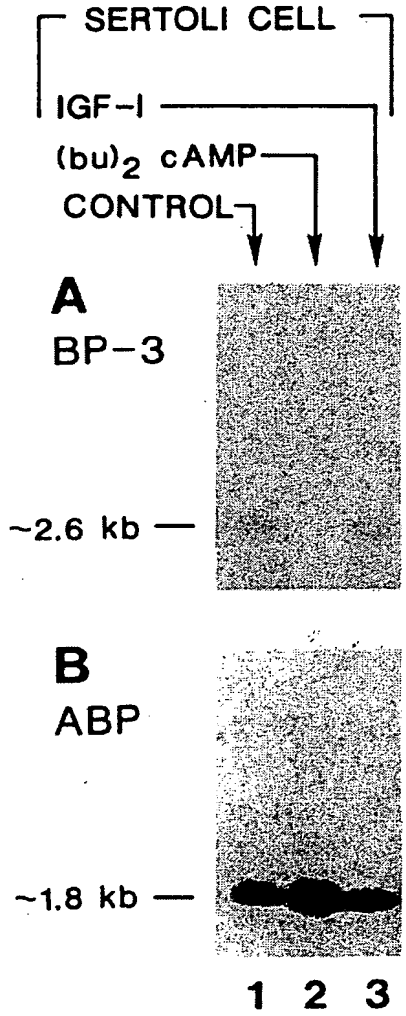

FIG. 1. Northern blot hybridization with either BP-3 cDNA (A) or androgen-binding protein (ABP) cDNA (B). Poly(A)$^+$ RNA was isolated from Sertoli cells treated for 48 h with 50 μM $(Bu)_2$cAMP, 100 ng/ml IGF-I, or no hormones (control). RNAs were electrophoresed, as described in *Materials and Methods*, and hybridized with BP-3 cDNA, followed by stripping and rehybridization with the ABP cDNA. Lane 1, Two micrograms, control; lane 2, 2 μg, 50 μM $(Bu)_2$cAMP; lane 3, 2 μg, 100 ng/ml IGF-I. Kilobase (kb) estimates are based on relative migration compared to λ DNA markers (Bethesda Research Laboratories, Gaithersburg, MD).

Effects of coincubation with $(Bu)_2$cAMP and IGF-I

We next examined the effects of coinbubation of IGF-I and $(Bu)_2$cAMP. Because the effect of $(Bu)_2$cAMP involved a major reduction in mRNA abundance, $(Bu)_2$cAMP, through presumed effects on BP-3 synthesis, might dominate over IGF-I. The effect of prior incubation with $(Bu)_2$cAMP, followed by coincubation with $(Bu)_2$cAMP, on the dose response to IGF-I was tested on BP-3 expression in the medium at 48 h (Fig. 2). $(Bu)_2$cAMP reduced, but did not eliminate, the actions of IGF-I to increase BP-3 in a dose-responsive manner. Twenty-four hours of prior incubation with $(Bu)_2$cAMP reduced the baseline level further, but did not eliminate the IGF-I dose response.

Effects of insulin

To investigate the IGF-I induction of BP-3 further, micromolar concentrations of insulin were tested for their capacity to increase BP-3 abundance in the medium. If the effects of IGF-I on BP-3 were secondary to IGF receptor-mediated events, concentrations of insulin that cross-react with the IGF receptors should mimic the effects of IGF-I (32). Insulin, at micromolar concentrations, did not consistently increase BP-3 levels (Fig. 3). To be certain that effective concentrations of insulin were maintained under the experimental conditions, medium insulin concentrations were measured by RIA at the conclusion of an experiment. When the original concentration was 1 μg/ml, 477 ng/ml remained, indicating that adequate amounts of insulin were indeed present in the medium and that insulin degradation did not account for the lack of an observed effect.

To confirm that insulin was interacting with the IGF-I receptor at the concentrations examined, crude plasma membrane preparations of Sertoli cells from day 15 primary cultures were affinity cross-linked with [^{125}I]IGF-I in the presence of graded concentrations of unlabeled IGF-I, IGF-II, or insulin. Micromolar concentrations of insulin displaced labeled IGF-I from the binding subunit of the IGF-I receptor (Fig. 4). IGF-I displaced labeled IGF-I at nanomolar concentrations. Low concentrations of IGF-II were without effect, although in experiments not shown, IGF-II appears to increase BP-3 concentrations in conditioned medium at concentrations comparable to those of IGF-I.

Effects of IGF analogs on BP-3

The lack of an insulin effect and the absence of changes in mRNA in response to IGF-I suggested that changes in

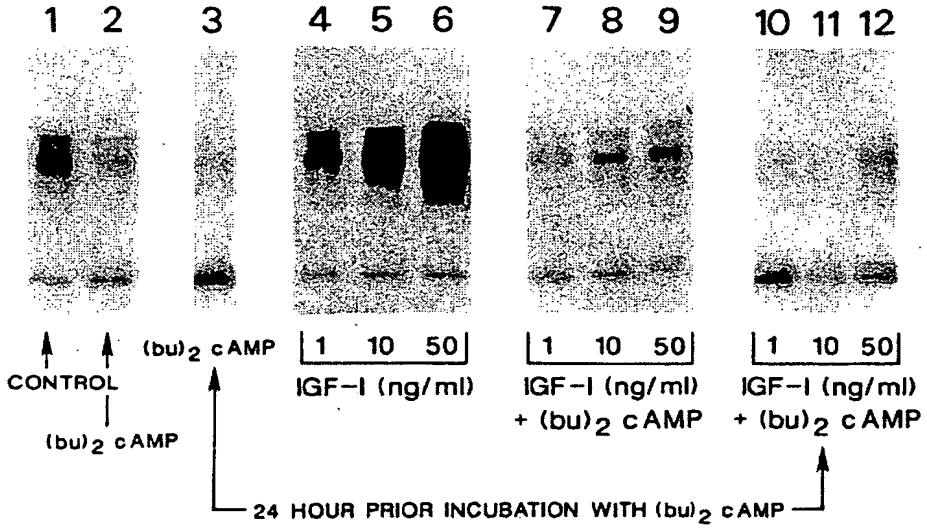

FIG. 2. Ligand blot analysis of BP-3 expression in cultured rat Sertoli cells; effect of prior incubation with $(Bu)_2$cAMP and coincubation of $(Bu)_2$cAMP and IGF-I. On day 5 of culture, Sertoli cells were changed to fresh serum-free medium containing either $(Bu)_2$cAMP (50 μM) or 0.001% BSA. After 24 h, medium was collected and replaced with fresh medium containing 0.001% BSA, $(Bu)_2$cAMP, IGF-I at graded concentrations, or $(Bu)_2$cAMP coincubated with IGF-I at graded concentrations. At 48 h, the medium was collected, concentrated, and processed, as described in *Materials and Methods*. Lane 1, Control; lane 2, $(Bu)_2$cAMP alone; lane 3, $(Bu)_2$cAMP [24 h prior incubation with $(Bu)_2$cAMP); lanes 4–6, IGF-I at graded concentrations; lanes 7–9, $(Bu)_2$cAMP (50 μM) and IGF-I at graded concentrations; lanes 10–12, same as lanes 7–9, but with prior incubation with $(Bu)_2$cAMP$_2$. The slower migrating doublet represents BP-3.

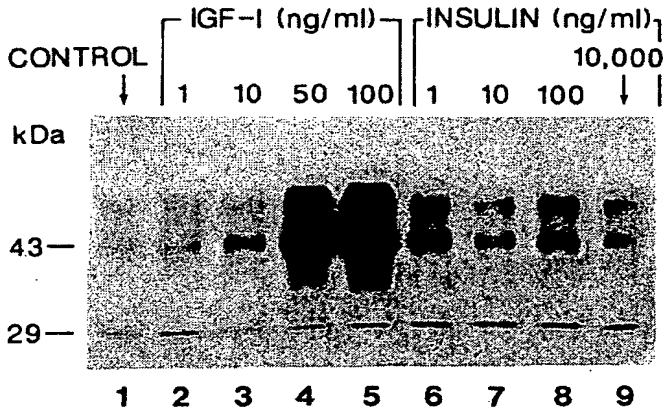

FIG. 3. Ligand blot analysis of BP-3 expression in cultured rat Sertoli cells; comparison of dose response to IGF-I *vs.* insulin. On day 5 of culture, Sertoli cells were changed to fresh medium containing IGF-I (10.0–100.0 ng/ml final concentration) or insulin (1.0–10,000 ng/ml final concentration). Medium was collected from triplicate dishes at 48 h, pooled, and processed, as described in *Materials and Methods*. The positions of ^{14}C-labeled mol wt markers (Bethesda Research Laboratories) are indicated in kilodaltons on the *left*.

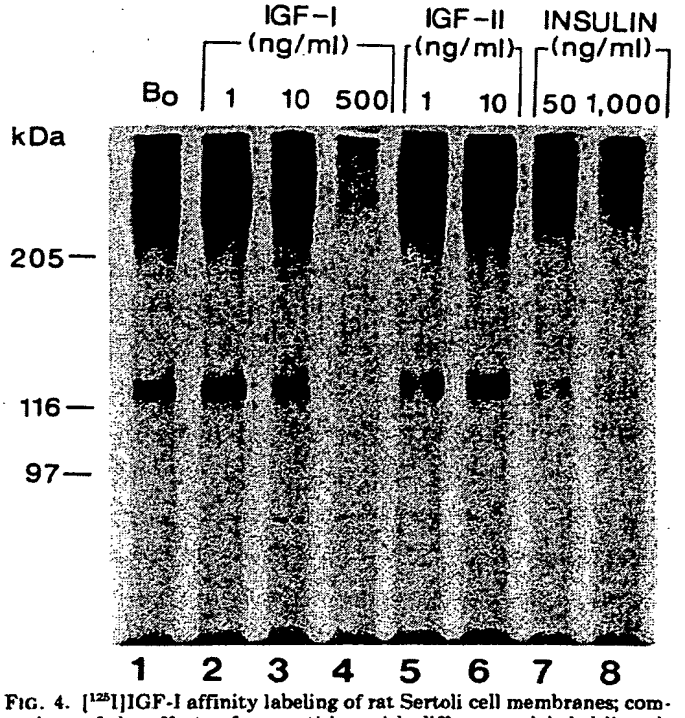

FIG. 4. [^{125}I]IGF-I affinity labeling of rat Sertoli cell membranes; comparison of the effects of competition with different unlabeled ligands (IGF-I, IGF-II, and insulin). Five hundred micrograms of crude Sertoli cell membranes were incubated with 500,000 cpm [^{125}I]IGF-I and graded concentrations of the different ligands, affinity labeled with disuccinimidyl suberate, electrophoresed, and subjected to autoradiography, as described in *Materials and Methods*. B_o, Labeling in the absence of unlabeled hormone.

synthesis might not account for the increased BP-3 observed in IGF-I-treated cells. The next series of experiments addressed whether the effects might be related to interaction of IGF with BP-3. Analogs of IGF-I that possess either altered affinity for the IGF-I receptor or altered affinity to BP-3 were tested for their capacity to induce an increase in BP-3 (20–22). Initially, to confirm the relative affinities of the human analogs for rat receptors and BPs, the analogs were subjected to competitive binding studies with either soluble BP from Sertoli cell-conditioned medium or to a IGFBP-deficient membrane preparation from the rat neuroblastoma cell line

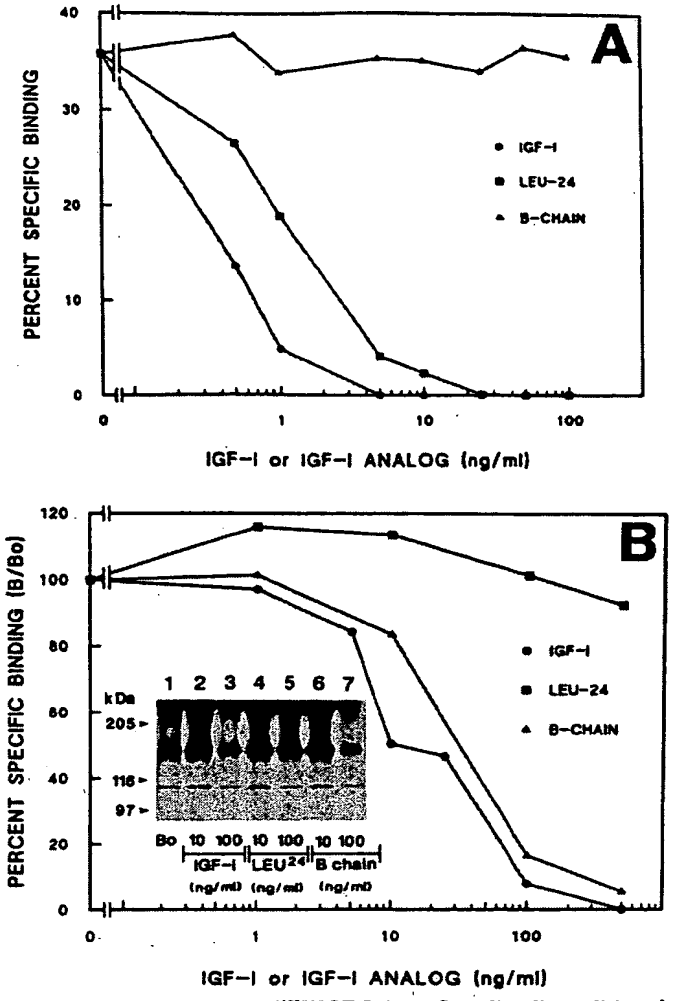

FIG. 5. A, Displacement of [^{125}I]IGF-I from Sertoli cell-conditioned medium BPs, as determined by charcoal assay. [^{125}I]IGF-I (20,000 cpm) were incubated at 4 C overnight with 60 μl Sertoli cell-conditioned medium in the presence of graded amounts of unlabeled IGF-I, B-chain mutant, or Leu$_{24}$ and processed, as described in *Materials and Methods*. B, Displacement of [^{125}I]IGF-I from B104 membranes by unlabeled IGF-I, Leu^{24}, or B-chain mutant. B104 membranes were incubated with [^{125}I]IGF-I in the presence of graded concentrations of unlabeled ligands. Aliquots were removed for affinity labeling, as described in *Materials and Methods*, electrophoresed, and subjected to autoradiography, as depicted in the panel within the figure. The remainder of the sample was centrifuged, and the amount of membrane-bound radioactivity in the pellet was counted. Percent specific binding was defined as the percentage of the total counts per min bound minus the percentage bound in the presence of an excess of unlabeled IGF-I.

B104 (29). Figure 5A shows that the B-chain mutant displayed minimal capacity to displace binding of [^{125}I]IGF-I to soluble Sertoli cell-BP, as determined by charcoal assay. In contrast, Leu^{24} was comparable to IGF-I under these conditions. However, when [^{125}I]IGF-I was incubated with B104 membranes in the presence of graded concentrations of IGF-I, B-chain mutant, or Leu^{24}, the B-chain mutant effectively displaced [^{125}I]IGF-I binding, whereas Leu^{24} was ineffective (Fig. 5B).

Having confirmed the expected relative affinities of the IGF-I analogs, their actions were tested on BP-3 expression in the cultured Sertoli cell (Fig. 6). Leu^{24}, despite minimal binding to the IGF-I receptor at the concentrations employed, increased BP-3 abundance; B-chain mutant had minimal efficacy. These data suggested that IGF-I receptor-mediated interactions do not contribute to regulation by IGF under these conditions.

Studies with recombinant BP-3

Because the IGF-I-induced increase in BP-3 appeared to be receptor independent and in some way related to the interaction of IGF-I with BP-3, experiments were performed to study the effect of IGF-I on changes in the abundance of exogenously added BP-3. Recombinant BP-3 was added to Sertoli cell cultures in the presence or absence of IGF-I, and the change in medium BP-3 abundance was measured by performing ligand blots and Western blots on aliquots of medium at different time points. As shown in Fig. 7, exogenously added rBP-3 at a starting concentration of 200 ng/ml was markedly reduced by 24 h of incubation. If rBP-3 was coincubated with IGF-I at 200 ng/ml, however, the reduction was less, with detectable BP-3 present after 48 h. Immunoblot analysis using antihuman BP-3 antiserum confirmed the identity and retention of the exogenously added rBP-3. At 48 h, there was no decrease if BP-3 was added to medium in a microfuge tube, as shown in the *last lane* of Fig. 7. As observed previously, endogenous BP-3 was increased by IGF-I and decreased by FSH compared to control values. If a response to graded amounts of IGF-I was achieved with a constant amount of BP-3, and BP-3 abundance determined at 24 h, the concentration of BP-3 was clearly greater in the

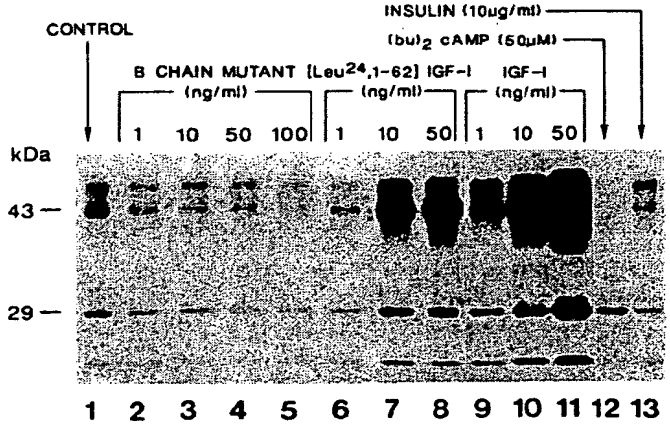

FIG. 6. Ligand blot analysis of BP-3 expression in cultured rat Sertoli cells; comparison of the effects of IGF-I and analogs of IGF-I with altered affinity for BP-3. On day 5 of culture, Sertoli cells were changed to fresh serum-free medium containing graded concentrations of B-chain mutant IGF-I (lanes 2–5), [Leu^{24},1–62]IGF-I (lanes 6–8), or IGF-I (lanes 9–11). Controls were no added growth factor (lane 1), (Bu)$_2$cAMP (50 μM; lane 12), or insulin (10 μg/ml; lane 13). Media were collected at 48 h and processed, as described in *Materials and Methods*.

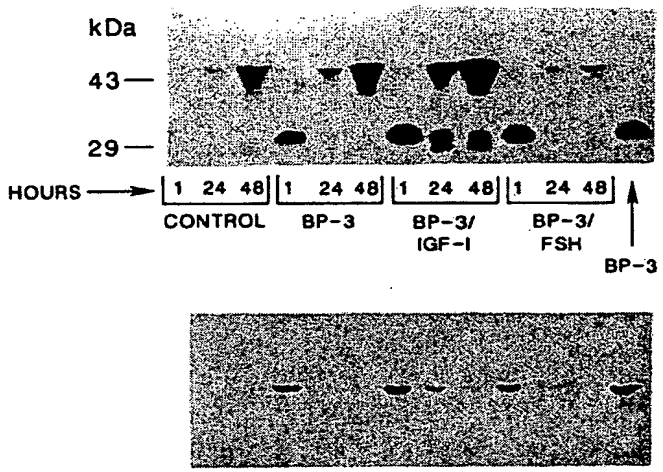

FIG. 7. Comparison by ligand and immunoblot analysis of the effects of IGF-I and FSH on endogenous BP-3 expression and exogenously added rBP-3 decay in cultured rat Sertoli cells. On day 5 of culture, Sertoli cells in 35-mm dishes were changed to 2.0 ml fresh 0.1% BSA medium containing BP-3 alone (200 ng/ml), BP-3 (200 ng/ml) with IGF-I (200 ng/ml), or BP-3 (200 ng/ml) with FSH (100 ng/ml). At 1, 24, and 48 h, 100-μl aliquots were removed from each duplicate dish, pooled, and transferred to a microfuge tube. Eighty microliters were electrophoresed for ligand blot analysis, followed by immunoblotting. The *last lane* represents a sample with BP-3 added, with the medium incubated in microfuge tube for 48 h rather than in the presence of Sertoli cells. The *upper panel* represents the ligand blot, and the *lower panel* represents the immunoblot.

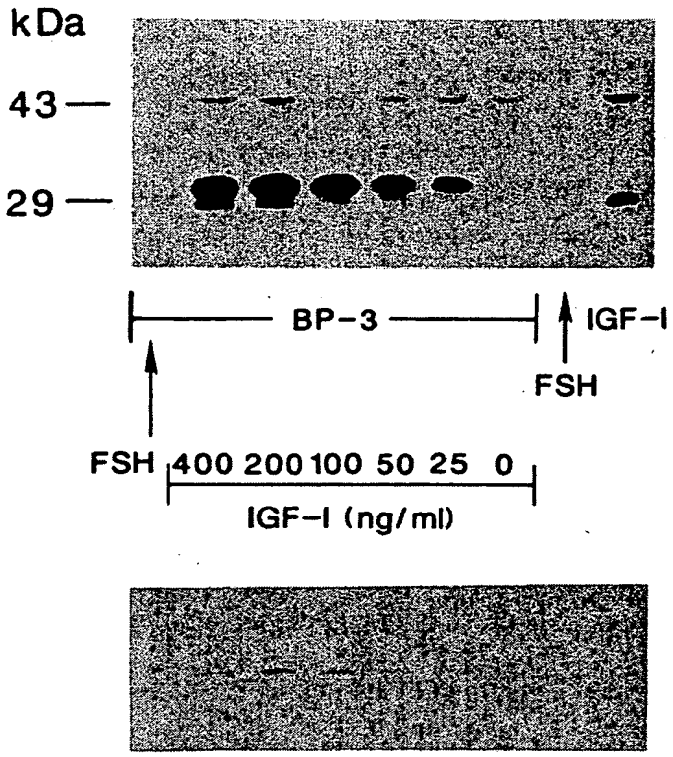

FIG. 8. Comparison by ligand/immunoblot analysis of the effects of graded concentrations of IGF-I and FSH on endogenous BP-3 expression and exogenously added rBP-3 decay. On day 5 of culture, Sertoli cells in duplicate 35-mm dishes were changed to fresh 0.1% BSA medium containing IGF-I alone (200 ng/ml; *last lane*), FSH alone (100 ng/ml; *next to last lane*), or BP-3 (200 ng/ml) with either graded concentrations of BP-3 or FSH (100 ng/ml). At 24 h, the medium was collected and pooled, and 80 μl were electrophoresed for ligand blot analysis, followed by immunoblotting. The *upper panel* represents the ligand blot, and the *lower panel* represents the immunoblot.

medium as the concentration of IGF-I increased to 200 ng/ml (Fig. 8) and then appeared to plateau.

To determine whether the interaction of IGF with glycosylated BP-3 also affects abundance in medium relative to affinity for BP-3, iodinated rgBP-3 was added to Sertoli cells in the presence of the different analogs. After 48 h, medium was collected, electrophoresed in 12% SDS-PAGE, and subjected to autoradiography (Fig. 9). In samples derived from medium that was treated with IGF-I or the Leu^{24} analog, greater amounts of radiolabeled BP-3 were detectable in the media. The B-chain mutant and [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I were no different from control or insulin values. This suggests again that interaction of BP-3 with IGF-I attenuates the decay of BP-3 from the medium.

To determine whether the decrease in medium BP-3 might be accounted for by a corresponding relative increase in cell-associated BP, Sertoli cells were incubated with BP-3 in the presence or absence of IGF-I as in previous experiments. Both medium and cell membranes were assayed by ligand blot for BP-3 abundance. As previously observed, the medium concentrations decreased rapidly without coincubation with IGF-I (Fig. 10, *upper row*). The corresponding membranes (*bottom row*) revealed proportionately more BP-3 compared to the BP-3/IGF-I combination, suggesting that contributing to the decrease in BP-3 is a significant difference in the cellular uptake of BP-3 when not complexed with IGF-I.

Discussion

The present study demonstrates that cAMP and IGF-I regulate BP-3 expression in cultured rat Sertoli cell-conditioned medium by divergent mechanisms. The effect of IGF is clearly independent of interaction of IGF-I with the IGF-I receptor, but, rather, involves the consequences of direct action of IGF-I with BP-3. The capacity of synthetic IGF-I analogs to enhance BP-3 accumulation appears solely related to their affinity for BP-3. The consequence of IGF-I interaction with BP-3 is a relative increase in the medium at least in part secondary to altered clearance of BP-3 by the Sertoli cell. In contrast, FSH or the cAMP agonist $(Bu)_2$ cAMP decreases BP-3 most likely by decreasing mRNA abundance, rather than by influencing the stability or clearance of BP-3.

The effects of IGF or insulin on BP-3 medium content have been studied in a number of different culture systems (33). In sparsely grown cultures of human fetal fibroblasts (34) and in human neonatal fibroblasts (35), IGF-I increases BP-3. Similar to our results, Clemmons *et al.* (36), using human fetal fibroblasts, showed not only that IGF-I at 24 h increases BP-3 content in conditioned medium by approximately 2.5-fold, but also that IGF analogs that do not have significant affinity for BP-3 do not increase BP-3, suggesting that a receptor-independent mechanism is involved. Further studies by Conover (37) have confirmed this phenomenon

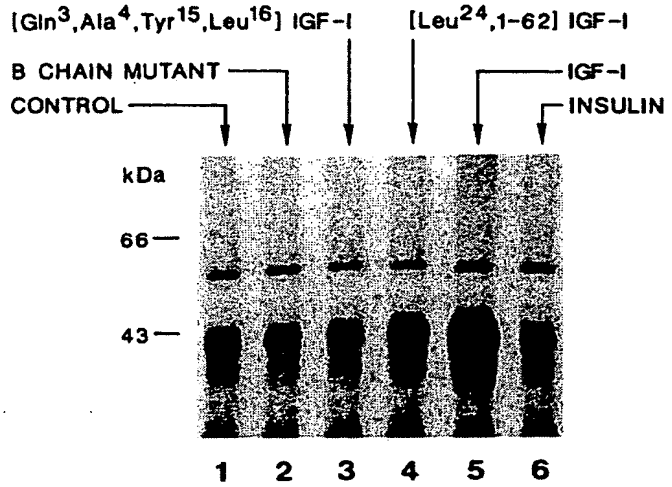

FIG. 9. Effects of IGF-I and IGF-I analogs on [^{125}I]rgBP-3 added to the medium of cultured Sertoli cells. On day 5 of culture, Sertoli cells were changed to fresh serum-free medium containing different growth factors at concentrations of 10 ng/ml. [^{125}I]rgIGFBP-3 (100,000 cpm) was added to each dish, followed by incubation for 48 h. Aliquots of conditioned medium were directly electrophoresed, dried, and subjected to autoradiography.

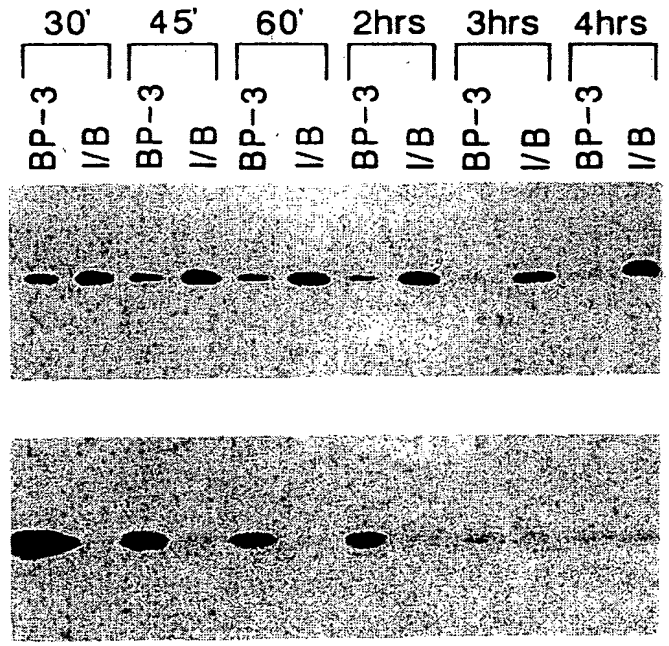

FIG. 10. Ligand blot analysis of the effects of IGF-I on the abundance of exogenously added rBP-3; comparison over time of medium (*upper row*) *vs.* cell-associated rBP-3 (*bottom row*). On day 5 of culture, Sertoli cells in duplicate 35-mm dishes were changed to fresh 0.1% BSA medium. At time zero, either BP-3 at a final concentration of 200 ng/ml or BP-3 with IGF-I (both at 200 ng/ml) were added to the dishes. At the indicated times, medium was collected, and membranes were processed, as described in *Materials and Methods*. Eighty microliters of medium and half of the microsomal membrane pellet were electrophoresed and processed for ligand blot analysis.

in the human fibroblast by demonstrating that a monoclonal antibody specific for the IGF-I receptor fails to block the IGF-I effect.

Our studies in the differentiated nondividing rat Sertoli cell extend studies of the fibroblast and demonstrate convincingly that the effect of IGF on BP-3 abundance is a direct result of IGF interaction with BP-3. In particular, we have shown that mRNA abundance is not changed with IGF treatment, the absolute effect is minimized when cAMP reduces the availability of BP-3, and the relative distribution of BP-3 in the medium *vs.* the cell surface is affected by IGF-I interaction with BP-3. Supporting the possibility that BP-3 is able to interact with the cell surface, Conover *et al.* (38) has shown that affinity labeling with [^{125}I]IGF-I in bovine fibroblasts preexposed to 10 nM bovine BP-3 results in the presence of a 50,000-dalton membrane-bound band that corresponds to BP-3, and recombinant human BP-3 associates with the cell surface when added to bovine fibroblasts (39). Although one possible effect in the fibroblast of BP-3 is to up-regulate IGF-I receptor abundance, increasing the sensitivity to IGF-I (39, 40), our studies suggest that an additional effect of the interaction of IGF-I with BP-3 may be to retard a clearance pathway; the presence or absence of IGF-I may influence the rate of this clearance.

Another possibility is that IGF-I binding protects BP-3 from degradation. Third trimester pregnancy serum appears to contain BP-3 proteolytic activity (41), raising the possibility that degradation pathways exist in the Sertoli cell. In the bovine fibroblast, Conover *et al.* (39, 40) has suggested that recombinant BP-3 is processed to smaller forms associated with the cell surface. The BP-3 in Sertoli cell medium appears quite stable, however, and is not subject to degradation when incubated at 37 C with conditioned medium derived from cells incubated with 0.1% BSA, FSH, or $(Bu)_2$cAMP (results not shown), and in addition, smaller BP-3 fragments are not detected by ligand or immunoblot. Although Sertoli cells may under some conditions degrade BP-3, this does not appear to account for the differences in content in the presence and absence of IGF-I, because fragments are not detected by immunoblot under these circumstances.

It is interesting to compare these *in vitro* results with recent studies focused on BP-3 regulation *in vivo* in plasma. Plasma levels of BP-3 are clearly increased by GH in plasma (42–44). *In vivo*, human IGF-I increases the abundance of the BP-3 doublet in the blood of either diabetic or hypophysectomized rats (45, 46), suggesting that the effect of GH on BP-3 may be indirect through IGF-I. Whether the effect of IGF-I on BP-3 is secondary to an effect on BP-3 synthesis *vs.* an effect on clearance or turnover is unclear given the evidence of protease activity for BP-3 under some conditions (41) and the apparent lack of an effect of IGF-I on synthesis in most cultured cell conditions.

The dominance of cAMP inhibition over IGF stimulation in the tissue culture environment can be explained in light of the two distinct mechanisms involved. The undetectable levels of BP-3 mRNA in $(Bu)_2$cAMP-treated Sertoli cells suggest a marked reduction in BP-3 synthesis related to decreased BP-3 mRNA abundance. IGF-I, in contrast, has no obvious effect on synthesis, but, rather, alters the properties of secreted BP-3 to influence its uptake by the Sertoli cell.

Thus, when the production of BP-3 is severely limited by cAMP, as occurs with FSH exposure or $(Bu)_2$cAMP, IGF-I has only modest effects. As might be predicted given the primary role of FSH in regulating Sertoli cell function, FSH would appear to be a principal determinant of BP-3 expression in the cultured Sertoli cell.

Recent analysis of the putative human BP-3 promoter identifies potential SP-1- and AP-2-binding domains, but fails to identify serum or classical cAMP response elements (CRE) (47). The CREs may be within intron sequences or further 5′ within the BP-3 promoter; however, there are genes that have shown to be acutely regulated through AP-2 elements (48). In addition, proteins related to CREB, an activator of the CRE, have been cloned which, rather than activate, appear to down-regulate cAMP transcription mediated through CREs (49). This or related genes may ultimately account for the actions of FSH on BP-3 expression in the Sertoli cell. Equally likely, cAMP responsiveness may be secondary to *trans*-acting tissue-specific factors that bind to unique regulatory motifs. For example, in a different cell, the UMR 106–01 rat osteoblast-like cell line, cAMP has no effect on BP-3 medium abundance, but, rather, has a marked stimulatory effect on 29/24-kilodalton BP concentrations (50) supporting the possibilities of tissue-specific regulatory factors. Ultimately, the cultured rat Sertoli cell may prove to be an excellent model system to investigate tissue-specific CREs.

The physiological significance of the contrasting effects of FSH and IGF-I are not clear. Although the action of IGF would appear to be controlled globally at the level of IGF-I synthesis, how the local concentrations and effective biological activity are modulated to serve the requirements for control of hormone action is still largely speculative. In the testis, it is likely that local IGF biological activity is regulated in a tissue-specific manner by pituitary gonadotropins. Similar to what has been reported in the ovary (16, 51, 52), BP-3 expression may provide a mechanism by which the IGF-mediated actions of FSH are modulated. In the seminiferous tubule, an unusually compartmentalized structure, BP-3, by virtue of its effect on reducing clearance to the Sertoli cell, may act as a carrier of IGF-I to neighboring spermatogenic cells. Our findings indicate that it is possible that in the intact testis, the interplay between FSH and locally synthesized testicular IGF-I combine to regulate the concentrations and tissue distribution of BP-3. The integrated effect may be to exquisitely control IGF biological activity.

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Structural Determinants for Binary and Ternary Complex Formation between Insulin-like Growth Factor-I (IGF-I) and IGF Binding Protein-3*

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Structural analogs of recombinant human insulin-like growth factor-I (IGF-I), with alterations to each of the B, C, A, and D domains, have been tested for their ability to form binary complexes with IGF-binding protein-3 (IGFBP-3) and ternary complexes with IGFBP-3 and the acid-labile subunit (α-subunit). Two functionally distinct regions of IGF-I have been identified. The first, involving residues 3 and 4 and the α-helix between residues 8 and 18 of the B-domain, as well as residues 49–51 in the A-domain, appears important for IGFBP-3 binding, such that substitution of these residues results in decreased binary complex available for α-subunit binding. The second region, distal to the IGFBP-3-binding epitope and primarily involving the D-domain and B-domain near residue 24, with some involvement of the C-domain, appears slightly inhibitory to binary complex formation, such that analogs with a truncated D-domain or with a Gly_4 bridge substituted for the C-domain show enhanced binding to IGFBP-3. However, binary complexes formed from these analogs bind the α-subunit with reduced affinity, the effect being most marked when substitution of the C-domain, or replacement of Tyr^{24}, is superimposed on D-domain truncation. It is concluded that although the α-subunit does not itself bind IGF-I, its interaction with IGFBP-3 in the ternary complex is dependent on structural determinants on IGF-I distal to the IGFBP-3 binding domain.

Peptides of the insulin-like growth factor (IGF)[1] family circulate predominantly as part of ternary complexes of approximately 140 kDa, comprising an acid-labile glycoprotein of ~85 kDa (α-subunit), an acid-stable glycoprotein of 40–50 kDa, IGF-binding protein-3 (β-subunit), and either IGF-I or IGF-II (γ-subunit) (1–6). IGF-binding protein-3 (IGFBP-3) appears central to this complex, with a separate high affinity binding site ($K_a = 2–3 \times 10^{10}$ M^{-1}) for IGFs (7) and lower affinity binding site ($K_a = 4–6 \times 10^8$ M^{-1}) for the α-subunit (5). The total concentration of IGFs in serum is approximately equimolar with that of IGFBP-3 (8), consistent with the observation that bound forms of IGFs have greatly extended circulating half-lives compared with free IGFs (9). In contrast, the α-subunit circulates in a 2–3-fold molar excess over the other components of the ternary complex; this may help to explain why most of the IGFBP-3 is in the ternary form, despite the low affinity constant for α-subunit binding (5, 8, 10).

The two binding sites on IGFBP-3 are functionally related. Whereas the presence of α-subunit has no effect on the interaction of IGF-I or IGF-II with the IGF binding site (5), the interaction of α-subunit with its binding site requires the presence of an IGF (5, 6). Thus there are no unoccupied IGF binding sites in the circulating 140-kDa complex since α-β dimers are unable to form. Little else is known of the factors affecting α-subunit binding although it is clear that there is a major charge component to the interaction, which is strongly inhibited by high ionic strength and polyanions such as heparin (11). The regulation of ternary complex formation is likely to be an important component of the overall regulation of IGF action since circulating IGFs are thought to be unable to cross the capillary barrier when in the ternary complex but are able to reach the tissues when in binary complexes with IGFBPs (12, 13).

Although the presence of IGF is required for α-subunit binding to IGFBP-3, IGF binding site occupancy is not the only determinant of α-subunit binding. For example, whereas IGF-II binds to IGFBP-3 with a higher affinity than IGF-I (7), α-subunit binds with higher affinity to the β-γ complex containing IGF-I than to that containing IGF-II (5). To gain a more detailed understanding of the structural requirements for ternary complex formation, we have employed a series of IGF-I analogs (14–17) with defined mutations affecting each of the four domains (B, C, A, and D) of the IGF-I structure (18). These studies indicate that binding affinity of the α-subunit for β-γ complexes is strongly dependent on the structure of the IGF in the complex, with determinants in each of the IGF-I domains.

MATERIALS AND METHODS

Peptides and Proteins—Natural human IGF-I was isolated from Cohn fraction IV of human plasma (19) and iodinated with $Na^{125}I$ as in previous studies (7). Recombinant human IGF-I and the following IGF-I analogs were prepared, expressed, purified, and characterized as described previously. B-domain variants were [Gln^3,Ala^4]IGF-I, [Tyr^{15}, Leu^{16}]IGF-I, [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I, Phe^{-1},Val^1,Asn^2,Gln^3,His^4,Ser^5,His^9,Glu^{12},Tyr^{15},Leu^{16}]IGF-I (B-chain mutant) (15), and [Ser^{24}] IGF-I (14). A-domain variants were [Ile^{41},Glu^{45},Gln^{46},Thr^{49},Ser^{50},Ile^{51},Ser^{53},Tyr^{55},Gln^{56}]IGF-I (A-chain mutant), [Thr^{49}, Ser^{50},Ile^{51}]IGF-I, and [Tyr^{55},Gln^{56}]IGF-I (16); C- and D-domain variants were [1–62]IGF-I, [1–27,Gly_4,38–70]IGF-I, [1–27, Gly_4,38–62] IGF-I (17), and [Leu^{24},1–62]IGF-I. IGFBP-3 was isolated from Cohn

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein.

fraction IV of human plasma (7) and the α-subunit from human serum (5), as in previous studies; ^{125}I-labeled α-subunit was prepared and purified by ion-exchange chromatography as described previously (10).

Binding Studies—Binding of IGF-I analogs to IGFBP-3 was measured by competition with ^{125}I-labeled IGF-I, essentially as described previously (5, 7). In brief, incubations in 0.3 ml of 50 mM sodium phosphate, 2.5 g/liter bovine albumin (Sigma, radioimmunoassay grade), pH 6.5, contained 0.5 ng of pure IGFBP-3, ^{125}I-labeled IGF-I (approximately 10,000 cpm, 25 pg), and IGF-I analogs tested over the concentration range 0.01–100 ng/0.3 ml. After 2 h at 22 °C, bound tracer was separated from free by incubation for 4 h at 22 °C with 0.5 μl of anti-IGFBP-3 antiserum R-7, and precipitation in the presence of 2.5 μl of goat anti-rabbit immunoglobulin (Bioclone, Sydney, Australia) and 4.5% final concentration polyethylene glycol 6000 (Merck) as described previously (5).

Ternary complex formation in the presence of different IGF-I analogs was measured by the binding of ^{125}I-labeled α-subunit (approximately 10,000 cpm, 1 ng) to mixtures of IGFBP-3 (10 ng) and IGF-I analogs, exactly as described previously (11), except that the IGF-I analogs were tested over the concentration range 0.05–100 ng/0.3 ml. Bound tracer was separated from free by immunoprecipitation as described above, with 1 μl of antiserum R-7 and 5 μl of goat anti-rabbit immunoglobulin. To study the IGFBP-3 dose dependence of ternary complex formation, an identical protocol was used, except that the IGF-I analog concentration was held constant at 10 ng/0.3 ml, and the IGFBP-3 concentration was varied over the range 0.5–50 ng/0.3 ml. Because of the higher IGFBP-3 concentrations used, 2.5 μl of antiserum R-7 and 10 μl anti-rabbit immunoglobulin were required for immunoprecipitation.

The association constant for α-subunit binding to β-γ (*i.e.* IGFBP·IGF) complexes was determined as described above, except that the concentrations of IGFBP-3 and the IGF-I analogs were held constant at 10 ng/0.3 ml. Unlabeled pure human α-subunit was generally added over the concentration range 10–400 ng/0.3 ml, although for some curves the 400-ng point was omitted. Bound and free tracer were separated with 1 μl of antiserum R-7 and 5 μl of anti-rabbit immunoglobulin. Nonspecific binding, measured as radioactivity immunoprecipitated in the absence of IGFBP-3, was subtracted from total binding before Scatchard plots were constructed. Data were fitted by linear regression, and differences between binding parameters were evaluated by Scheffé's test after analysis of variance, performed using Statview SE+Graphics (Abacus Concepts, Berkeley, CA).

RESULTS

The Effect of B-domain Mutations—Fig. 1 shows the competition for ^{125}I-labeled IGF-I binding to IGFBP-3 by IGF-I and IGF-I variants altered in the B-domain. Similar curves for IGF-I were obtained whether the natural or recombinant peptide was used, with half-maximal displacement by 0.19 ± 0.06 ng of IGF-I/0.3 ml reaction mixture (S.D., n = 5). [Gln^3,Ala^4]IGF-I and [Tyr^{15},Leu^{16}]IGF-I gave similar competition curves, nonparallel with the IGF-I curve and somewhat lower in activity than IGF-I. In contrast, the curve for [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I was parallel to that for IGF-I; in three experiments, the relative activity was lower than that of IGF-I by a factor of 110 ± 30 (S.D.). The B-chain mutant, tested at up to 100 ng/0.3 ml, was unable to compete with ^{125}I-labeled IGF-I for binding to IGFBP-3. Mutations of Tyr^{24} were tested by comparing [Ser^{24}]IGF-I with intact IGF-I (Fig. 1), and the carboxyl-terminally truncated mutant, [Leu^{24},1–62]IGF-I, with the corresponding IGF-I analog, [1–62]IGF-I. Data for [Leu^{24},1–62]IGF-I are shown below, under "The Effect of C- and D-domain Mutations." For both IGF-I and [1–62]IGF-I, analogs substituted at residue 24 gave displacement curves that were approximately parallel to the corresponding unsubstituted analog and reduced in relative IGFBP-3 binding activity by about 2-fold.

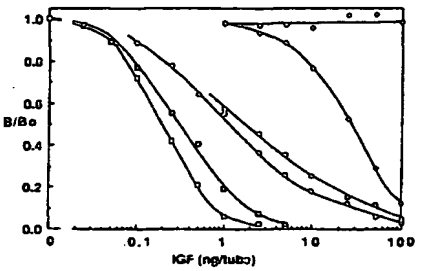

FIG. 1. **Competition for the binding of ^{125}I-labeled IGF-I to human IGFBP-3 (0.5 ng) by B-domain mutants of IGF-I.** The analogs shown are IGF-I (□), [Gln^3,Ala^4]IGF-I (○), [Tyr^{15},Leu^{16}]IGF-I (○), [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I (◇), [Ser^{24}]IGF-I (□), and B-chain mutant (◇). B/B_0 represents the ratio of radioactivity bound in the presence of IGF analogs to that bound in the absence of unlabeled ligand.

Fig. 2*a* shows IGF dose-response curves for ternary complex formation, *i.e.* α-subunit binding to binary complexes. In five experiments, half-maximal α-subunit binding was seen at an IGF-I concentration of 0.26 ± 0.15 ng/0.3 ml (S.D.). The binding of α-subunit to the β-γ complex containing [Tyr^{15},Leu^{16}]IGF-I consistently occurred less readily than to the complex containing [Gln^3,Ala^4]IGF-I although their binding to IGFBP-3 appeared similar. Interestingly, α-subunit binding in the presence of the latter peptide was virtually identical to that seen in the presence of IGF-I, over the entire range of IGF concentrations (Fig. 2*a*). For this reason and because of limited supplies of [Gln^3,Ala^4]IGF-I, no further ternary complex formation studies were performed with this peptide. [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I gave a dose-response curve for α-subunit binding parallel to that for IGF-I, but 100-fold lower in apparent activity, consistent with the lower binding of this analog to IGFBP-3. In the presence of B-chain mutant little or no α-subunit binding was seen, as expected from its lack of binding to IGFBP-3. The dose-response curves for [Ser^{24}]IGF-I and [Leu^{24},1–62]IGF-I both reached a plateau at 30–40% of the α-subunit binding seen with the corresponding peptides lacking the residue 24 substitution (not shown). IGFBP-3 dose-response curves for B-domain variants are shown in Fig. 2*b*. In three experiments, half-maximal α-subunit binding in the presence of IGF-I was seen at an IGFBP-3 concentration of 1.9 ± 0.2 ng/0.3 ml (S.D.). The curves obtained in the presence of [Tyr^{15},Leu^{16}]IGF-I and [Ser^{24}]IGF-I were parallel to that seen with IGF-I but reduced in apparent activity by 2–5-fold. Similarly, α-subunit binding in the presence of [Leu^{24},1–62]IGF-I showed parallelism with that for [1–62]IGF-I, with a 5-fold decrease in apparent activity. In contrast, [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I gave a non-

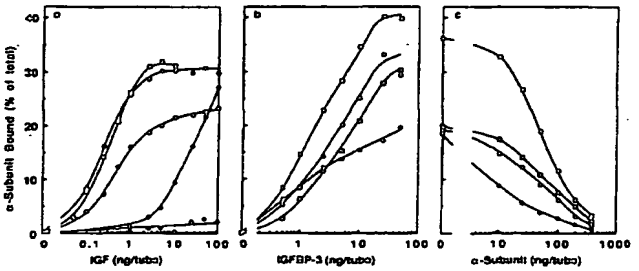

FIG. 2. **Formation of the ternary IGF·IGFBP complex in the presence of B-domain mutants of IGF-I.** The analogs shown are IGF-I (□), [Gln^3,Ala^4]IGF-I (○), [Tyr^{15},Leu^{16}]IGF-I (○), [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I (◇), [Ser^{24}]IGF-I (□), and B-chain mutant (◇). *Panel a*, ^{125}I-labeled α-subunit binding to 10 ng of IGFBP-3 in the presence of increasing IGF concentrations. *Panel b*, ^{125}I-labeled α-subunit binding to increasing IGFBP-3 concentrations in the presence of 10 ng of each IGF analog. *Panel c*, competition by increasing concentrations of unlabeled α-subunit for ^{125}I-labeled α-subunit binding to 10 ng of IGFBP-3 in the presence of 10 ng of IGF analog.

parallel curve when analyzed in this way. This analysis was not performed on [Gln^3,Ala^4]IGF-I or the B-chain mutant.

The effect of competition for ^{125}I-labeled α-subunit binding by increasing α-subunit concentrations in the presence of B-domain variants is shown in Fig. 2*c*. As expected from the IGFBP-3 dose-response curves shown in Fig. 2*b*, α-subunit tracer binding to 10 ng of IGFBP-3 in the absence of unlabeled α-subunit was much lower in the presence of [Tyr^{15},Leu^{16}]IGF-I, [Ser^{24}]IGF-I, or [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I. The addition of increasing unlabeled α-subunit competed for tracer binding to the β-γ complex containing [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I much more effectively than to complexes containing IGF-I or the other B-domain variants.

Scatchard plots for the influence of B-domain variants on ternary complex formation are shown in Fig. 3, and summary data are presented in Table I. Assuming that α-subunit only binds to binary (β-γ) complexes (5, 6) and that α-subunit binding has no effect on the IGF-IGFBP-3 interaction (5), the binding site concentration derived from Scatchard plots is a measure of the binary complex concentration. In the presence of intact IGF-I (natural or recombinant), the calculated association constant for α-subunit binding was 1.12 ± 0.07 × 10^9 M^{-1} (S.D., n = 5), and the mean binding site concentration was 0.59 ± 0.08 nM. If the association constant for IGF-I binding to IGFBP-3 is 2 × 10^{10} M^{-1} (7) and the molar concentrations of IGFBP-3 and IGF-I (both 10 ng/0.3 ml) are 0.775 and 4.39 nM, respectively (molecular masses of 43 and 7.6 kDa, respectively), it can be calculated that the binary complex concentration at equilibrium should be 0.764 nM; that is, 98.6% of IGFBP-3 has IGF-I bound to it. The calculated binding site concentration from Scatchard analysis therefore represents 0.77 ± 0.10 mol of α-subunit bound per mol of binary complex. The slightly higher binding affinity compared with values reported previously (5, 11) probably results from the use of an α-subunit preparation with slightly increased potency in the present study.

[Tyr^{15},Leu^{16}] substitution decreased the apparent binding site concentration (*i.e.* binary complex concentration) by more than 40%, with a small decrease in binding affinity also seen (Fig. 3*a* and Table I). However, the low α-subunit binding in the presence of [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I was entirely a result of a major decrease in apparent binding sites (*i.e.* reduced binary complex), with the affinity actually increasing significantly. These effects contrast with that of Tyr^{24} substitution, as shown in Fig. 3, *b* and *c*: for both intact IGF-I and [1–62]IGF-I, substitution of this residue resulted in a 50% decrease in apparent affinity, with no significant effect on apparent binding site concentration (Table I).

The Effect of A-domain Mutations—Substitutions in the A-domain of IGF-I had relatively minor effects of binary or ternary complex formation. Fig. 4 shows that whereas the A-chain mutant and [Thr^{49},Ser^{50},Ile^{51}]IGF-I were 2–4-fold less effective than IGF-I in displacing ^{125}I-labeled IGF-I from IGFBP-3, [Tyr^{55},Gln^{56}]IGF-I was similar in activity to IGF-I. The slightly reduced binding activity of the A-chain mutant and [Thr^{49},Ser^{50},Ile^{51}]IGF-I was reflected in their reduced activity in the ternary complex formation assays (Fig. 5, *a* and *b*). In contrast, [Tyr^{55},Gln^{56}]IGF-I was the least active of these analogs in ternary complex formation, despite its normal activity in binary complex formation, suggesting the involvement of residues 55 and 56 in α-subunit binding. Scatchard analysis, illustrated in Fig. 6 and Table I, indicated that these substitutions significantly lowered the binding affinity for α-subunit and confirmed the lack of effect on binding sites (*i.e.* binary complex formation).

The Effect of C- and D-domain Mutations—These mutations consisted of substitution of the C-domain of IGF-I by a Gly_4 bridge ([1–27,Gly_4,38–70]IGF-I) or truncation of the D-domain ([1–62]IGF-I). Analogs with either mutation had 2–3-fold increased activity in binary complex formation whereas the presence of both mutations increased the activity by 4-fold over that of IGF-I (Fig. 7). As described above, substitution of Tyr^{24} decreased the activity of [1–62]IGF-I by approximately 2-fold, with [Leu^{24},1–62]IGF-I similar to IGF-I (Fig. 7). Despite high activity in the binary complex assay, D-domain deletion decreased ternary complex formation, as

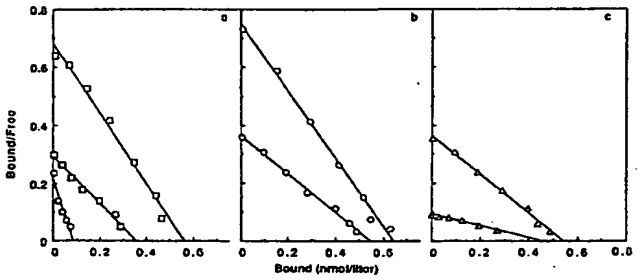

FIG. 3. **Scatchard plots of α-subunit binding to IGFBP-3 in the presence of B-domain mutants of IGF-I.** The analogs shown are IGF-I (□), [Tyr^{15},Leu^{16}]IGF-I (□), and [Gln^3,Ala^4,Tyr^{15},Leu^{16}] IGF-I (○) (*panel a*); IGF-I (○) and [Ser^{24}]IGF-I (○) (*panel b*); and the carboxyl-terminally truncated analogs [1–62]IGF-I (△) and [Leu^{24},1–62]IGF-I (△) (*panel c*).

TABLE I

Binding parameters for the formation of ternary complexes among α-subunit, IGFBP-3, and various structural analogs of IGF-I

Ternary complex formation was measured by the binding of radiolabeled α-subunit to mixtures of IGFBP-3 and IGF-I analogs, as described under "Materials and Methods." Values are means ± S.D. for n determinations.

IGF-I analog	Association constant	Binding site concentration	n
	nM^{-1}	nM	
IGF-I	1.124 ± 0.073	0.593 ± 0.079	5
[Tyr^{15},Leu^{16}]IGF-I	0.829 ± 0.019[a]	0.319 ± 0.036[a]	3
[Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I	1.483 ± 0.086[b]	0.116 ± 0.002[b]	3
[Ser^{24}]IGF-I	0.605 ± 0.093[b]	0.541 ± 0.061	3
[1-62]IGF-I	0.623 ± 0.079[b]	0.510 ± 0.028	3
[Leu^{24},1-62]IGF-I	0.291 ± 0.118[b,c]	0.330 ± 0.108[b]	4
[Tyr^{55},Gln^{56}]IGF-I	0.803 ± 0.134[a]	0.536 ± 0.100	3
[1-27,Gly_4,38-70]IGF-I	0.882 ± 0.030	0.610 ± 0.095	3
[1-27,Gly_4,38-62]IGF-I	0.378 ± 0.079[b]	0.531 ± 0.022	3

[a] Significant difference from value for IGF-I, $p < 0.05$.
[b] Significant difference from value for IGF-I, $p < 0.01$.
[c] Significant difference from value for [1-62]IGF-I, $p < 0.05$.

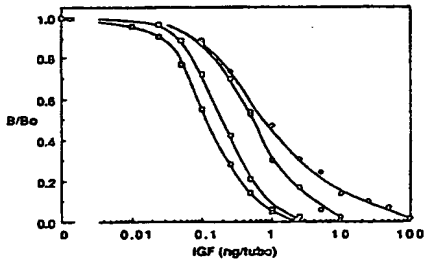

FIG. 4. **Competition for the binding of ^{125}I-labeled IGF-I to human IGFBP-3 (0.5 ng) by A-domain mutants of IGF-I.** The analogs shown are IGF-I (□), A-chain mutant (○), [Thr^{49},Ser^{50},Ile^{51}] IGF-I (◇), and [Tyr^{55},Gln^{56}]IGF-I (□). B/B_0 represents the ratio of radioactivity bound in the presence of IGF analogs to that bound in the absence of unlabeled ligand.

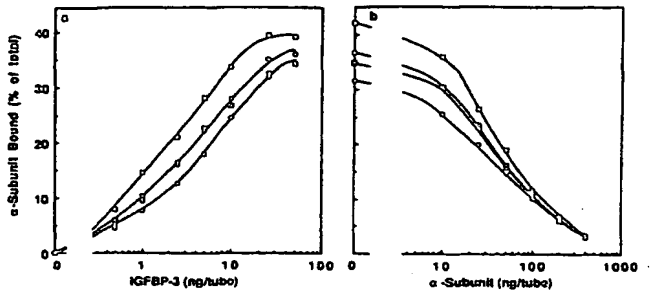

FIG. 5. **Formation of the ternary IGF·IGFBP complex in the presence of A-domain mutants of IGF-I.** The analogs shown are IGF-I (▩), A-chain mutant (□), [Thr^{49},Ser^{50},Ile^{51}]IGF-I (○), and [Tyr^{55},Gln^{56}]IGF-I (◎). *Panel a*, ^{125}I-labeled α-subunit binding to increasing IGFBP-3 concentrations in the presence of 10 ng of each IGF analog. *Panel b*, competition by increasing concentrations of unlabeled α-subunit for ^{125}I-labeled α-subunit binding to 10 ng of IGFBP-3 in the presence of 10 ng of IGF analog.

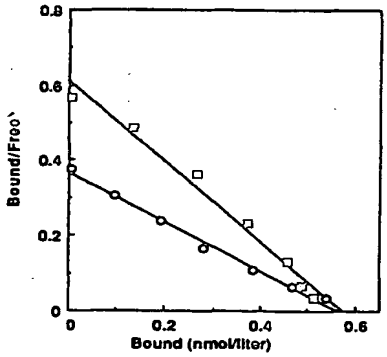

FIG. 6. **Scatchard plots of α-subunit binding to IGFBP-3 in the presence of IGF-I (□) and the A-domain IGF-I mutant [Tyr^{55},Gln^{56}]IGF-I (○).**

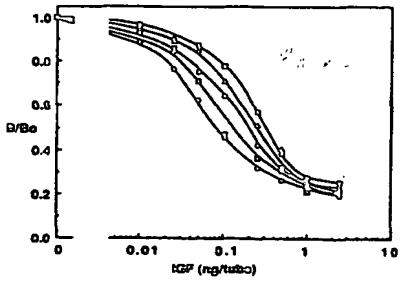

FIG. 7. **Competition for the binding of ^{125}I-labeled IGF-I to human IGFBP-3 (0.5 ng) by C- and D-domain mutants of IGF-I.** The analogs shown are IGF-I (▩), [1–62]IGF-I (□), [Leu^{24},1–62]IGF-I (△), [1–27,Gly$_4$,38–70]IGF-I (◎), and [1–27,Gly$_4$,38–62]IGF-I (○). B/B_0 represents the ratio of radioactivity bound in the presence of IGF analogs to that bound in the absence of unlabeled ligand.

shown in Fig. 8, with the C-domain substitution imposing an additional effect. Binding of α-subunit, measured over a range of IGF concentrations, was lower in the presence of [1–62]IGF-I than that seen with IGF-I, with [1–27, Gly$_4$,38–62]IGF-I giving still lower binding (Fig. 8*a*). The same order of apparent activity was reflected in the IGFBP-3 dose-response curves (Fig. 8*b*) and the α-subunit competitive binding curves (Fig. 8*c*). Fig. 8 (*a–c*) also illustrates the fact that substitution of Tyr^{24} for leucine, when superimposed on D-domain truncation, severely increased the impairment in ternary complex formation.

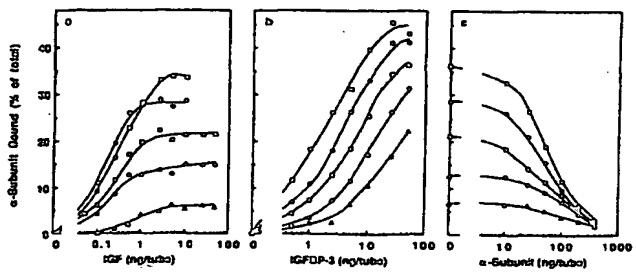

FIG. 8. **Formation of the ternary IGF·IGFBP complex in the presence of C- and D-domain mutants of IGF-I.** The analogs shown are IGF-I (▩), [1–62]IGF-I (□), [Leu^{24},1–62]IGF-I (△), [1–27,Gly$_4$,38–70]IGF-I (◎), and [1–27,Gly$_4$,38–62]IGF-I (○). *Panel a*, ^{125}I-labeled α-subunit binding to 10 ng of IGFBP-3 in the presence of increasing IGF concentrations. *Panel b*, ^{125}I-labeled α-subunit binding to increasing IGFBP-3 concentrations in the presence of 10 ng of each IGF analog. *Panel c*, competition by increasing concentrations of unlabeled α-subunit for ^{125}I-labeled α-subunit binding to 10 ng of IGFBP-3 in the presence of 10 ng of IGF analog.

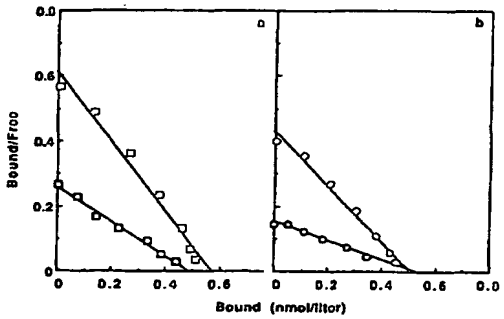

FIG. 9. **Scatchard plots of α-subunit binding to IGFBP-3 in the presence of C- and D-domain mutants of IGF-I.** The analogs shown are IGF-I (▩) and [1–62]IGF-I (□) (*panel a*); [1–27,Gly$_4$,38–70]IGF-I (○) and [1–27,Gly$_4$,38–62]IGF-I (○) (*panel b*).

When viewed as Scatchard plots (Fig. 9 and Table I), the decreased ternary complex formation of the C- and D-domain mutants is seen to be caused entirely by a decrease in α-subunit binding affinity. D-domain truncation decreased this affinity by almost 50% for intact IGF-I (Fig. 9*a*) and by almost 60% for C-domain-substituted IGF-I (Fig. 9*b*) whereas the effect of C-domain substitution itself was not statistically significant. As shown in Fig. 3*c*, a considerable additive effect of Tyr^{24} substitution on D-domain truncation was observed, resulting in a significantly greater loss of α-subunit binding affinity.

DISCUSSION

The IGFs differ from their structural analog insulin in that they bind to soluble binding proteins in addition to their membrane-bound receptors. Three structurally distinct classes of IGFBP have been studied extensively (20, 21), and six classes are now known to exist (22). Of these IGFBPs, only IGFBP-3 has been shown to interact additionally with an acid-labile glycoprotein, the α-subunit, to form ternary complexes (5, 6). The regulation of ternary complex formation may be an important factor in determining access of the IGFs from circulating complexes to their target tissues.

The IGF mutants used in this study have previously yielded information about the structural determinants on IGF-I involved in the interactions with the type 1 and type 2 IGF

receptors (14–17), the binding protein IGFBP-1 (23), and an impure preparation of acidified human serum in which the predominant IGFBP was thought to be IGFBP-3 (15, 24). The present report extends these findings by describing binding studies using a pure IGFBP-3 preparation and, more importantly, by characterizing the structural determinants on IGF-I which permit the interaction of IGFBP-3·IGF-I (β-γ) complexes with the α-subunit of the ternary complex. It has been demonstrated previously that IGF binding to IGFBP-3 is a prerequisite for α-subunit binding (5, 6), but it was not clear from these earlier studies whether IGF binding site occupancy was, *per se*, sufficient to allow α-subunit binding.

The results of binary complex formation studies presented here are in general agreement with those reported previously for the crude serum-binding protein preparation (14–17), consistent with the assumption that the main binding species in that preparation is IGFBP-3. To summarize these findings, [Tyr^{55},Gln^{56}]IGF-I and all of the C- and D-domain mutations had approximately 2–4-fold higher activity than IGF-I in binding to pure IGFBP-3 whereas [Gln^3,Ala^4]IGF-I, [Tyr^{15},Leu^{16}]IGF-I, [Ser^{24}]IGF-I, A-chain mutant, and [Thr^{49},Ser^{50},Ile^{51}]IGF-I were approximately 2–10-fold less active than IGF-I. The B-chain mutant showed no binding activity to IGFBP-3, as described for the serum IGFBP preparation (15); however, [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I consistently showed a 100-fold reduced activity in binding to pure IGFBP-3, in contrast to the 600-fold reduced activity reported for the serum IGFBP preparation (15). This discrepancy suggests that a binding protein other than IGFBP-3, which may be unable to bind [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I, contributes to the total IGF binding capacity of the crude serum preparation.

Based on the model of the tertiary structure of IGF-I proposed by Blundell and co-workers (25, 26), it may be concluded that residues 3–4, 49–51, and 55–56 are on the surface of the IGF-I molecule in locations suitable for interaction with IGFBP-3. The α-helical region of the B-domain (residues 8–18) also plays a critical role. These epitopes are on the opposite surface of IGF-I from the C- and D-domains (25, 26). One possible explanation for the enhanced IGFBP-3 binding in analogs lacking these domains is that a conformational change induced in IGF-I, when bound to IGFBP-3, might move the C- and D-domains into a slightly unfavorable configuration, which would be avoided in the deletion mutants.

Although several of the peptides tested had a higher activity than IGF-I in binding to IGFBP-3, none was more active in allowing ternary complex formation, *i.e.* the binding of the α-subunit to the β-γ complex. Of the B-domain mutants tested, [Gln^3,Ala^4]IGF-I was unique in that its ability to allow α-subunit binding appeared identical to that of IGF-I, although it bound to IGFBP-3 with 5–10-fold lower activity than IGF-I. This may indicate that the removal of Glu^3 from IGF-I, while decreasing IGFBP-3 binding (as also seen with the IGF-I analog lacking residues 1–3 (27)), induces a conformational change in IGFBP-3 which encourages α-subunit binding. For all of the other B-domain mutants tested, the degree of inhibition of α-subunit binding seen in their presence reflected their weaker binding to IGFBP-3, relative to IGF-I. Thus the B-chain mutant was entirely inactive in both the binary and ternary complex formation assays, [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I had a 100-fold lower activity than IGF-I in both assays, while [Tyr^{15},Leu^{16}]IGF-I had severalfold lower activity than IGF-I in both assays. Scatchard analysis in the presence of these peptides indicated that the decreased ability of IGFBP-3 to bind α-subunit was largely caused by an apparent decrease in binding site concentration, *i.e.* a decreased formation of the binary complex which is required for α-subunit binding. This was seen most clearly for [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I, in the presence of which the available α-subunit binding sites on IGFBP-3 were decreased by over 80% compared with IGF-I.

Of the A-domain mutants, [Thr^{49},Ser^{50}, Ile^{51}]IGF-I and the A-chain mutant resembled the B-domain mutants in that the decrease in their ability to allow ternary complex formation reflected their slightly lower ability to bind to IGFBP-3. This, then, appears to be the consequence of mutations that directly affect the IGFBP-3-binding epitope of IGF-I. In contrast, replacement of the arginine residues at positions 55 and 56 of IGF-I did not decrease binding affinity for IGFBP-3 but decreased affinity for ternary complex formation. Since these two arginine residues are absent in the analogous positions of IGF-II, this may explain the slightly reduced activity of IGF-II for ternary complex formation (5).

The D-domain analogs, despite enhanced binding to IGFBP-3 when compared with IGF-I, appeared deficient in causing the conformational change in IGFBP-3 necessary for α-subunit binding. When analyzed by Scatchard plot, this was reflected in a decrease in the binding affinity for α-subunit. Substitution of Tyr^{24} exacerbated the decrease in α-subunit binding caused by D-domain deletion. Therefore, the conformation of the D-region and residue 24 of IGF-I appears to be somehow involved in α-subunit binding. Although direct binding studies provide no evidence for interaction between the α-subunit and the IGFs in the absence of IGFBP-3 (5, 6), it was postulated previously on the basis of affinity crosslinking studies that in the presence of IGFBP-3, a region of IGF-I comes in close proximity (~10 Å) to the α-subunit (6). Our data now suggest that the region concerned may involve the D-domain and the region surrounding residue 24 of the B-domain.

In summary, these studies have defined two regions of the IGF-I molecule involved in the formation of the ternary complex among the IGF, IGFBP-3, and the α-subunit. The first, involving the helical region of the B-domain and residues at positions 3, 4, and 49–51, appears to be important in IGFBP-3 binding such that when this binding is reduced, there is less binary complex available for the interaction with the α-subunit. The second, distal to the IGFBP-3-binding epitope and primarily involving the D-domain and B-domain near residue 24, appears to be slightly inhibitory to IGFBP-3 binding but may, after IGFBP-3 binding has occurred, be required for the interaction of the α-subunit with the IGF·IGFBP-3 complex. These studies may lead the way to the design of bioactive IGF-I analogs which, while binding to IGFBP-3, are entirely unable to participate in ternary complex formation. Such analogs would provide unique information as to the biological significance of the ternary complex as a transporter of IGFs and a regulator of their cellular access.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 057491/0413

In re patent application of
MASCARENHAS

Group Art Unit: 1654

Serial No. 09/399,120

Examiner: Anish GUPTA

Filed: September 20, 1999

For: NULL IGF FOR THE TREATMENT OF CANCER

DECLARATION UNDER 37 CFR § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

I, Andreas Sommer, hereby declare that:

1. I am a principal scientist at INSMED, the assignee of the captioned application. I have worked in the field of protein chemisty, growth factor biology, including the study of the insulin-like growth factor family (IGFs), and preclinical and clinical protein drug development. I have published over 40 papers in this field. My qualifications are set out in my *curriculum vitae*, which is attached hereto as APPENDIX A.

2. I have reviewed and understood the subject application and the final office action dated November 5, 2002. As set out in detail below, it is my opinion that, based on the instant specification, one of skill in the art would be able to recognize the therapeutic potential of a null IGF, identify the null IGFs suitable for practicing the methods of the present invention, and establish appropriate protocols for using such null IGF compounds in the treatment of a cancer.

3. It is my understanding that the PTO is contending that none of the null IGF compounds disclosed in the instant invention are enabled for slowing the growth rate or progression of cancer, including prostate cancer. It is also my understanding that, based on the examiner's comments during an interview dated February 26, 2003, the Office doubts the predictive value of therapeutic data in a PC-3 xenograft animal model. In particular, I understand that the Office is of the opinion

that the PC-3 animal model cannot sufficiently mimic human prostate cancer and therefore cannot be used to predict the therapeutic utility of a null IGF.

4. The null IGF technology described in the present invention takes advantage of IGF-I analogs that have lower affinity for an IGF-I receptor than a wild-type IGF, yet bind the major IGF binding protein-3 (IGFBP-3) with equal, nearly equal, or better affinity that the wild-type IGF. In this regard, a [Leu 60] IGF (*i.e.*, Y60L), for example, can be used to slow wild-type IGF-induced cellular growth, including tumor cell growth, and improve the survival rate. This is demonstrated in the PC-3 xenograft model used in the present invention.

5. Moreover, it would be reasonable to expect null IGF analogs, such as [Ala31, Leu60] IGF-I; [Leu24, Leu60] IGF-I; [Leu24, Ala31, Leu60] IGF-I; [Leu24, 59, 60, Ala31] IGF-I; [1-27, Gly4, 38-70] IGF-I; [Ser24] IGF-I; [Leu24, 1-62] IGF-I and [1-29, gly, gly, gly, gly, 42-62] IGF-I, to also have anti-cancer benefits based on the data provided in the instant specification. These analogs meet the criteria of reduced binding to the IGF-I receptor and normal or nearly normal binding to IGFBP-3.

6. Other ideal null IGF analogs can be obtained by conservative amino acid changes at any position in the C and/or D domain in an IGF-I molecule. Indeed, the experimental results based on [Leu60] IGF-I that are presented in the instant application clearly indicate that other null IGF analogs that share the same properties as [Leu60] IGF-I would result in the same beneficial effects.

7. It is my belief that the data from the PC-3 xenograft animal model used in the instant invention will predict the therapeutic utility of the above-specified IGF-I analogs in a human cancer. As the invention clearly identifies the molecular target as wild-type IGF, which activates the IGF-I receptor and promotes cellular growth, the described null IGF technology acts to attenuate the growth effects of circulating natural IGFs. Thus, the ability of null IGF to decrease cell growth is not model dependent.

8. Furthermore, recent literature has been presented which implicates various insulin-like growth factors in a variety of carcinomas, including breast, lung, prostate, colorectal and other

cancers. Therefore, as predicted from studies in the PC-3 animal model, a null IGF applied to any cancer associated with IGF-induced cellular proliferation would have an anti-cancer effect.

8. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Andreas Sommer Date: 04/02/2003
Andreas Sommer, Ph.D.

1: Hum Gene Ther 2002 Apr 10;13(6):761-73 Related Articles, Links

p53 and PTEN/MMAC1/TEP1 gene therapy of human prostate PC-3 carcinoma xenograft, using transferrin-facilitated lipofection gene delivery strategy.

Seki M, Iwakawa J, Cheng H, Cheng PW.

Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, 984525 Nebraska Medical Center, Omaha, NE 68198, USA.

We previously reported that supplementation of a cationic liposome with transferrin (Tf) greatly enhanced lipofection efficiency (P.-W. Cheng, Hum. Gene Ther. 1996;7:275-282). In this study, we examined the efficacy of p53 and PTEN tumor suppressor gene therapy in a mouse xenograft model of human prostate PC-3 carcinoma cells, using a vector consisting of dimyristoyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE)-cholesterol (DC) and Tf. When the volume of the tumors grown subcutaneously in athymic nude mice reached 50-60 mm(3), three intratumoral injections of the following four formulations were performed during week 1 and then during week 3: (1) saline, (2) DC + Tf + pCMVlacZ, (3) DC + Tf + pCMVPTEN, and (4) DC + Tf + pCMVp53 (standard formulation). There was no significant difference in tumor volume and survival between group 1 and group 2 animals. As compared with group 1 controls, group 3 animals had slower tumor growth during the first 3 weeks but thereafter their tumor growth rate was similar to that of the controls. By day 2 posttreatment, group 4 animals had significantly lower tumor volume relative to initial tumor volume as well as controls at the comparable time point. Also, animals treated with p53 survived longer. Treatment with DC, Tf, pCMVp53, DC + pCMVp53, or Tf + pCMVp53 had no effect on tumor volume or survival. Expression of p53 protein and apoptosis were detected in tumors treated with the standard formulation, thus associating p53 protein expression and apoptosis with efficacy. However, p53 protein was expressed in only a fraction of the tumor cells, suggesting a role for bystander effects in the efficacy of p53 gene therapy. We conclude that intratumoral gene delivery by a nonviral vector consisting of a cationic liposome and Tf can achieve efficacious p53 gene therapy of prostate cancer.

PMID: 11936974 [PubMed - indexed for MEDLINE]

☐ 2: Prostate 2000 Jul 1;44(2):133-43 Related Articles, Links

Metastatic burden in nude mice organs measured using prostate

tumor PC-3 cells expressing the luciferase gene as a quantifiable tumor cell marker.

Rubio N, Villacampa MM, El Hilali N, Blanco J.

Department of Cell Biology, Institut de Recerca Oncologica, Barcelona, Spain.

BACKGROUND: Sensitive procedures for quantitative measurement of tumor cell spread as a function of time and primary tumor size are necessary to generate models of metastasis and formulate therapies. METHODS: Prostate carcinoma cells PC-3.luc expressing the luciferase gene were intramuscularly inoculated in nude mice to generate experimental tumors. Metastatic cells in target organs were easily counted by their capacity to produce light. RESULTS: Tumor cells were very mobile and migrated to all the target organs examined: lymph nodes, brain, bone, lungs, liver, kidney, spleen, testicles, prostate, seminal vesicle, and scrotum. Organ colonization started very early, 14 days after inoculation, when primary tumors were very small and produced an amount of light equivalent to that generated by 2 x 10(4) tumor cells in vitro (tumor cell equivalents, TCEs). Tumor cell burden could be quantitatively described by power functions of time or primary tumor light-producing capacity. The ratio of metastatic TCEs to primary tumor TCEs clustered around organ characteristic values: 10(-3) for femur and lumbar lymph nodes, 10(-6) for the spleen, and 10(-3) for the added set of organs. CONCLUSIONS: Dispersal of PC-3 tumor cells from IM experimental tumors started early before the third week postinoculation and when primary tumors had 2 x 10(4) TCEs. Tumor cells were found widely spread in all the organs tested. The possibility of easily quantifying tumor cell burden should make this approach useful for the study of metastasis and the development of antimetastatic therapies. Copyright 2000 Wiley-Liss, Inc.

PMID: 10881023 [PubMed - indexed for MEDLINE]

☐ 3: Lab Invest 1998 Oct;78(10):1315-25 Related Articles, Links

Traffic to lymph nodes of PC-3 prostate tumor cells in nude mice visualized using the luciferase gene as a tumor cell marker.

Rubio N, Villacampa MM, Blanco J.

Cell Biology Department, Consejo Superior de Investigaciones Cientificas, Barcelona, Spain.

Tumor cell traffic between intramuscular tumors experimentally induced in nude mice and lymph nodes was studied using PC-3.luc prostate adenocarcinoma cells permanently transfected with the luciferase gene as a tumor cell marker. This sensitive approach allowed the detection of 1 luminescent tumor cell mixed with 1

x 10(7) unlabeled PC-3 cells and of 1 tumor cell/lymph node. PC-3.luc cells inoculated in nude mice showed a 1000-fold expansion, accompanied by a 4.5-fold increase in tumor cell density (tumor cell number/gram of tumor), during the first 90 days of primary tumor growth. No macroscopic secondary tumors were found in organs, other than lymph nodes, by the end of the experiment. Tumor cell spread to lymph nodes was detected at Day 21, when there were 2 x 10(5) tumor cells at the inoculation sites, before discrete primary tumors could be identified. The total tumor cell burden in the tested lymph nodes was modeled by a power function of primary tumor cell number (determination coefficient R2 = 0.9472). By the end of the experiment, on Day 110, there were 1.8 metastatic cells in the studied lymph nodes for every 1000 primary tumor cells. These results suggest that empirically obtained tumor-specific indexes could be used to characterize the invasion of lymph nodes by tumor cells. The path of spread for PC-3.luc cells from intramuscular sites appears to follow the lymphatic system, and at no time during the experiment were tumor cells found in blood. An upper limit of no more than 16 blood-circulating tumor cells was established for these experiments. The observation of tumor cells that were invading the lymphatic system from the onset of tumor growth but unable to establish secondary tumors in other organs emphasizes the potential of this procedure in studying the multi-step nature of metastasis.

PMID: 9800957 [PubMed - indexed for MEDLINE]

☐ 4: Anticancer Res 1997 Nov-Dec;17(6D):4253-8 Related Articles, Links

Comparative intraosseal growth of human prostate cancer cell lines LNCaP and PC-3 in the nude mouse.

Soos G, Jones RF, Haas GP, Wang CY.

Department of Urology, State University of New York Health Science Center, Syracuse 13210, USA.

BACKGROUND: More than 75% of patients with advanced prostate carcinoma have skeletal involvement, which is the principal metastatic site and the major complication of this disease. The goal of this work was to compare the osseous metastasis of androgen-sensitive and insensitive prostate cancers in the nude mouse. MATERIALS AND METHODS: Androgen-sensitive LNCaP or -insensitive PC-3 human prostate carcinoma cells were injected directly into the femur medullas of male nude Beige mice, the animals were then sacrificed at successive time intervals to study the gross and microscopic characteristics of the established tumors. RESULTS: LNCaP and PC-3 both colonized in the bone marrow within a week, then gradually expanded to the entire bone medulla followed by osseous infiltration to produce obvious symptoms in the affected extremities. Based on the morphology, both osteoblastic and osteolytic changes

occurred during the course of tumor progression. In addition, PC-3 tumors eventually broke through the bone cortex, invaded the surrounding tissues, and metastasized to the regional lymph nodes. In contrast, LNCaP remained localized within the bone, and appeared to eventually regress and die after displacing the normal bone marrow cells. Immunohistochemically, LNCaP tumors were consistently positive for prostate-specific antigen in bone metastasis, while PC-3 tumors were negative. Tumor cell nuclei of both PC-3 and LNCaP hybridized to a human repeated sequence DNA probe indicating that the proliferating malignant cells were of human origin. CONCLUSIONS: These cancer cell lines produced a high incidence of growth in the bone that differed in histogenesis. The relative malignancy of these cell lines was demonstrated in this model.

PMID: 9494517 [PubMed - indexed for MEDLINE]

☐ 5: Prostate 1998 Feb 15;34(3):169-74 Related Articles, Links

Surgical orthotopic implantation allows high lung and lymph node metastatic expression of human prostate carcinoma cell line PC-3 in nude mice.

An Z, Wang X, Geller J, Moossa AR, Hoffman RM.

AntiCancer Inc., San Diego, California 92111, USA.

BACKGROUND: Prostate cancer is the second leading cause of male death in the United States. When diagnosed, nearly half the cases have metastatic lesions. An animal model of human prostate cancer demonstrating spontaneous metastasis from the orthotopic site after tumor implantation should be of great help for us to understand the disease and to formulate treatment strategy. We report here a high metastatic model of human prostate cancer PC-3. METHODS: We developed microsurgical techniques, termed surgical orthotopic implantation (SOI), to implant histologically intact tumor tissues orthotopically in immunodeficient mice. In this study intact tissue of the human prostate cancer cell line PC-3, harvested from a subcutaneous tumor in a nude mouse, was implanted to the ventral lateral lobes of the prostate gland in a series of nude mice. Mice were sacrificed when found moribund, and autopsy and histology were performed subsequently. RESULTS: A high frequency of lymph node and lung metastasis was noted upon histological examination. The extensive and widespread lung metastasis following orthotopic implantation of PC-3 is, to the best of our knowledge, the first report in the literature. CONCLUSIONS: In contrast to orthotopic injection of cell suspensions, no multiple metastatic cell selection was necessary after SOI for significant expression of the metastatic potential of PC-3. We conclude that the stromal tissue architecture maintained in the implanted tumor played a critical role in tumor growth and progression.

PMID: 9492844 [PubMed - indexed for MEDLINE]

☐ 6: Prostate 1997 Aug 1;32(3):164-72 Related Articles, Links

Luteinizing hormone-releasing hormone antagonist Cetrorelix (SB-75) and bombesin antagonist RC-3940-II inhibit the growth of androgen-independent PC-3 prostate cancer in nude mice.

Jungwirth A, Galvan G, Pinski J, Halmos G, Szepeshazi K, Cai RZ, Groot K, Schally AV.

Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana, USA.

BACKGROUND: Hormones like bombesin (BN)/gastrin-releasing peptide (GRP) and luteinizing hormone-releasing hormone (LH-RH) and growth factors such as epidermal growth factor (EGF) might be involved in the relapse of prostate cancer under androgen ablation therapy. Interference with receptors for BN/GRP, LH-RH, or EGF might provide a therapeutic approach to inhibit tumor growth of androgen-independent prostate cancer. METHODS: LH-RH antagonist Cetrorelix (SB-75) and the BN/GRP antagonist RC-3940-II were tested for their effects on the growth of the androgen-independent PC-3 human prostate cancer cell line xenografted into nude mice. Tumor growth, serum hormone levels, and receptor concentrations for BN/GRP and EGF were measured. RESULTS: When the treatment was started, tumor volume in all groups was 70-80 mm3. After 4 weeks, tumor volume in the control animals injected with saline was 871 +/- 233 mm3 and that of animals treated with Cetrorelix only 197 +/- 61 mm3. The BN/GRP antagonist RC-3940-II also significantly reduced PC-3 tumor volume in nude mice to 122 +/- 20 mm3. The combination of Cetrorelix and RC-3940-II produced no additional inhibition. High-affinity receptors for EGF were detected in the tumor membranes and their number was significantly decreased after administration of Cetrorelix or RC-3940-II. CONCLUSIONS: These findings demonstrate that LH-RH antagonists and BN/GRP antagonists inhibit the growth of the androgen-independent prostate cancer cell line PC-3 in vivo. Both analogs may exert a direct inhibitory effect on tumor growth through a down-regulation of EGF receptors.

PMID: 9254895 [PubMed - indexed for MEDLINE]

☐ 7: J Surg Oncol 1996 Jul;62(3):194-200 Related Articles, Links

Antisense oligonucleotide intralesional therapy for human PC-3

prostate tumors carried in athymic nude mice.

Rubenstein M, Mirochnik Y, Chou P, Guinan P.

Division of Cellular Biology, Hektoen Institute for Medical Research, Chicago, Illinois 60612, USA.

Previously we reported hemorrhagic necrosis in human-derived PC-3 prostate tumors, in athymic nude mice, produced by the intralesional injection of antisense oligonucleotides (oligos) directed against mRNAs encoding transforming growth factor-alpha (TGF-alpha) and its target, the epidermal growth factor receptor (EGFR). We now describe our experience with these oligos in treating additional mice with various doses and modes of administration. During prolonged treatment, a dose-response effect was observed, with the optimal dosage consisting of the combination of 400 micrograms of each oligo. Although responses varied, based upon amount and how oligos were administered, we found that tumors were best treated when initially less than 156 mm3. Intralesional inoculations produced necrosis and yielded responses, ranging from complete response (CR) or cure to partial responses (PR) in 9 of 12 tumors treated with full dose (400 micrograms of each oligo) and 1 of 1 treated with 800 micrograms of each oligo, against a large tumor. Included among the 9 positive responses with full-dose administration were 2 tumors that regressed (one completely). A single tumor treated with twice (2X) the normal dosage (800 micrograms of each oligo) also regressed. A single tumor treated with half (1/2) dose (200 micrograms of each) progressed similar to controls, as did 3 of 12 treated with the full dose. Limited experience with ALZET diffusion pumps gave CR (1 of 3) or PR (2 of 3) in 100% of tumors treated (including one mouse cured of multiple tumors in a five day period). It appears that multiple inoculations consisting of 400 micrograms of each oligo is most effective against these tumors, particularly when administered against tumors of <156 mm3 in initial size.

PMID: 8667627 [PubMed - indexed for MEDLINE]

☐ 8: Prostate 1995 May;26(5):227-34 Related Articles, Links

Spontaneous metastasis of PC-3 cells in athymic mice after implantation in orthotopic or ectopic microenvironments.

Waters DJ, Janovitz EB, Chan TC.

Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA.

The ability of subcutaneous, prostatic, and nonprostatic intraabdominal organ microenvironments to influence local tumor growth and metastasis of PC-3

human prostate carcinoma cells in athymic mice was determined. Tumorigenesis and metastasis of PC-3 were evaluated 60 days after subcutaneous and intraprostatic (orthotopic) implantation of 5 x 10(5) PC-3 cells in 6-week-old, male athymic mice. Intraprostatic implantation of PC-3 cells resulted in paraaortic lymph node metastases in 10 of 10 (100%) mice with prostatic tumors, whereas metastases were present in only 2 of 9 (22%) mice after subcutaneous implantation. Next, we determined whether the urinary bladder (nonprostatic, urogenital microenvironment) or stomach (nonurogenital, intraabdominal microenvironment) would facilitate the metastasis of PC-3 cells in athymic mice. Tumorigenesis and metastasis were 100% after subserosal implantation of PC-3 cells within the wall of the urinary bladder (n = 6 mice). Subserosal implantation of PC-3 cells into the stomach wall (n = 7 mice) also resulted in tumor formation and metastasis to regional lymph nodes in 100% of mice. In all experiments, regional lymph nodes were the most frequent site of metastasis, regardless of implantation site. We conclude that tumor microenvironment factors responsible for the metastasis of PC-3 cells in athymic mice may not be organ-specific, since nonprostatic visceral microenvironments are sufficient for predictable metastasis. Use of these models may further our understanding of how tumor microenvironment modulates expression of the metastatic phenotype by human prostate carcinoma cells.

PMID: 7753708 [PubMed - indexed for MEDLINE]

☐ 9: Int J Cancer 1993 Dec 2;55(6):963-7 Related Articles, Links

Effect of somatostatin analog RC-160 and bombesin/gastrin releasing peptide antagonist RC-3095 on growth of PC-3 human prostate-cancer xenografts in nude mice.

Pinski J, Schally AV, Halmos G, Szepeshazi K.

Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, New Orleans, LA 70146.

Nude mice bearing xenografts of the androgen-independent human prostate-cancer cell line PC-3 were treated for 4 weeks with somatostatin analog RC-160, bombesin/gastrin-releasing peptide (GRP) antagonist (RC-3095), or the combination of both peptides. In the first experiment, treatment was started when the tumors measured approximately 10 mm3. Tumor volumes and weights were reduced by about 40% by RC-160 or RC-3095 administered by s.c. injections at doses of 100 micrograms/day/animal and 20 micrograms/day/animal respectively. The combination of RC-3095 with RC-160 did not further potentiate suppression of tumor growth, but histologically the ratio of apoptotic and mitotic indices was significantly higher in the groups treated with the combination than in the other groups. Serum gastrin levels were significantly reduced in all treated groups.

Therapy with RC-160 or the combination also significantly decreased serum growth-hormone levels. Specific high-affinity binding sites for bombesin, somatostatin and epidermal growth factor (EGF) were found on the tumor membranes. Receptors for EGF were significantly down-regulated by treatment with RC-3095, RC-160 and a combination of both analogs. Tumors from mice treated with RC-160 showed a significant increase in maximal binding capacity for somatostatin as compared with control tumors, demonstrating the absence of down-regulation. In the second experiment, treatment was started when the tumors were well developed and measured approximately 90 mm3. No significant reduction in volume, weight and growth rate of tumors was found in the groups treated with RC-160 or RC-3095. Our results suggest that somatostatin analog RC-160 and bombesin/GRP antagonist RC-3095 can inhibit the growth of androgen-independent prostate cancer when the therapy is started at an early stage of tumor development.

PMID: 7902829 [PubMed - indexed for MEDLINE]

☐ 10: Cell Mol Biol Res 1993;39(8):751-60 Related Articles, Links

Plasminogen activator and metalloprotease activities of Du-145, PC-3, and 1-LN-PC-3-1A human prostate tumors grown in nude mice: correlation with tumor invasive behavior.

Wilson MJ, Sinha AA.

VA Medical Center, Minneapolis, MN 55417.

Proteolytic enzymes are required to mediate tumor cell invasion of adjacent tissues and spread of primary tumors to distant sites. Our objective was to examine the activities and molecular forms of plasminogen activator (PA) and matrix metalloproteases (MP) in primary and secondary growths of SC tumors of three human prostatic cell lines (Du-145, PC-3, and 1-LN-PC-3-1A [1-LN], a subline of PC-3) grown in nude mice. The plasminogen activator activities were 1.7 +/- 1.3 (+/- SD), 6.2 +/- 2.8, and 11.5 +/- 4.2 for Du-145, PC-3, and 1-LN in primary SC tumors, respectively. Urokinase was the predominant molecular form of PA found in each tumor as determined from its molecular size (predominantly 54 kDa with a minor activity of 33 kDa) and sensitivity to amiloride. Prominent MP activities of approximately 68, 76, and 96 kDa as well as lesser activities of about 56, 59, 63, 84, 165, and 180 kDa were found in 1-LN tumors, whereas only less active MP of 59, 68, and 96 kDa were detected in the parental PC-3 cells. Du-145 tumors expressed MP activities of 59 and 96 kDa. Treatment of 1-LN tumor extracts with p-aminophenylmercuric acetate (APMA) significantly reduced the MP activities of 76 and 165 kDa while increasing activities of 56, 59, 65, 68, and 84 kDa. The 76 and 165 kDa MP activities thus appear to be prominent proenzyme forms of MP expressed in the 1-LN tumor. Secondary growths of

tumor were subsequently found near the site of initial injection of PC-3 and 1-LN cells following removal of the primary tumor. There was a 42% increase in PA activity in the PC-3 secondary tumors, but only an 8% increase in 1-LN secondary tumors. However, there was no difference in the activities or number of molecular forms of MP in extracts of PC-3 or 1-LN primary or secondary tumors. The substantial expression of MP activities in the more aggressive 1-LN subline of the human prostatic PC-3 cell line indicates that induction of certain MP may be an important regulatory event in prostate tumor progression.

PMID: 7951414 [PubMed - indexed for MEDLINE]

☐ 11: Prostate 1989;15(2):187-94 Related Articles, Links

Patterns of metastasis by the human prostate cancer cell line PC-3 in athymic nude mice.

Shevrin DH, Gorny KI, Kukreja SC.

Section of Medical Oncology, University of Illinois, Chicago, IL 60612.

Cells from the PC-3 human prostate cancer cell line were evaluated in athymic nude mice in order to determine the influence of size of the primary tumor and site inoculation on the incidence and pattern of metastasis. At autopsy, all organs, including the skeleton, were evaluated for metastasis. Subcutaneous injections resulted in metastases to the draining axillary lymph node and lungs (56% and 13%, respectively), and were correlated with size of the primary tumor. Tail vein injection resulted in a high incidence of lung metastasis, while injection into the peritoneal space, spleen, and seminal vesicles resulted in intraabdominal tumor growth, liver metastasis, and large tumors within the seminal vesicles, respectively. Skeletal metastases were not observed in any of the animals studied. We conclude that injection of PC-3 cells into various sites results in different patterns of metastasis, but may not constitute an entirely suitable animal model of human prostate cancer due to the lack of metastasis to the skeleton.

PMID: 2529482 [PubMed - indexed for MEDLINE]

☐ 12: J Urol 1982 Nov;128(5):1064-7 Related Articles, Links

Spontaneous metastasis of cells of the human prostate carcinoma cell line PC-3 in athymic nude mice.

Ware JL, Paulson DF, Mickey GH, Webb KS.

Spontaneous metastasis and extensive invasiveness were observed in athymic nude mice injected with human prostatic carcinoma cells of the PC-3 line or heterotransplants of nude mouse supported PC-3 tumor. In 3 experimental series, 60, 63 and 50 per cent of the nude mice receiving subcutaneous inoculations of PC-3 cells or tumor heterotransplants developed 1 or more lymphatic tumor metastases. Examination of metaphase-arrested cells recovered from the metastatic sites confirmed the tumor origin as human in each case. Cells recovered from 1 nude mouse supported subline, MPC-3-10, frequently exhibited double minute chromosomes in addition to the typical PC-3 chromosomal profile. These observations provide the foundation for a study of the relationship between prostate carcinoma cell characteristics and lymphatic metastasis in the nude mouse.

PMID: 7176035 [PubMed - indexed for MEDLINE]

☐ 13: Prostate 2002 Aug 1;52(3):173-82 Related Articles, Links

Inhibition of proliferation of PC-3 human prostate cancer by antagonists of growth hormone-releasing hormone: lack of correlation with the levels of serum IGF-I and expression of tumoral IGF-II and vascular endothelial growth factor.

Plonowski A, Schally AV, Letsch M, Krupa M, Hebert F, Busto R, Groot K, Varga JL.

Endocrine, Polypeptide, and Cancer Institute, Veterans Affairs Medical Center, New Orleans, Louisiana 70112-1262, USA.

BACKGROUND: Antagonists of growth hormone-releasing hormone (GHRH) such as JV-1-38 can inhibit androgen-independent prostate cancer directly by several mechanisms and/or indirectly by suppressing growth hormone/insulin-like growth factor-I (GH/IGF-I) axis. To shed more light on the mechanisms involved, the effects of JV-1-38 on PC-3 human prostate cancer were compared with those of somatostatin analog RC-160 in vivo and in vitro. METHODS: Nude mice bearing PC-3 tumors received JV-1-38 (20 microg), RC-160 (50 microg) or a combination of JV-1-38 and RC-160. The concentration of IGF-I in serum and the expression of mRNA for IGF-II and vascular endothelial growth factor (VEGF) in tumor tissue were investigated. RESULTS: In vivo, the final volume of PC-3 tumors treated with JV-1-38 was significantly lowered by 49% ($P < 0.01$), whereas RC-160 exerted only 30% inhibition (NS), compared with controls. Combined use of both compounds augmented tumor inhibition to 63% ($P < 0.001$). Serum IGF-I levels were decreased only in mice treated with RC-160. JV-1-38 suppressed mRNA for IGF-II in PC-3 tumors by 42%, whereas RC-160 alone or in combination with JV-1-38 caused a 65% reduction. JV-1-38 and RC-160 used as single drugs decreased the expression of VEGF by 50%, and their

combination caused a 63% reduction. In vitro, JV-1-38 inhibited the proliferation of PC-3 cells by 39%. This effect could be partially reversed by addition of IGF-I to the serum-free medium. RC-160 alone did not affect the PC-3 cell growth in vitro, but in combination with JV-1-38 it augmented the antiproliferative effect of the GH-RH antagonist to 72%. Exposure to JV-1-38 in vitro reduced the expression of mRNA for IGF-II in PC-3 cells by 55% but did not change VEGF mRNA levels, whereas RC-160 had no effect. CONCLUSIONS: The antiproliferative effect of JV-1-38 was not associated with the suppression of serum IGF-I and was only partially correlated with the expression of IGF-II and VEGF in PC-3 tumors, suggesting that other mechanisms play a role in the antitumor action of GHRH antagonists. Nevertheless, the stronger inhibition of tumor growth after combined treatment with JV-1-38 and RC-160 indicates that the interference with multiple local stimulatory factors leads to an enhanced inhibition of prostate cancer. Copyright 2002 Wiley-Liss, Inc.

PMID: 12111694 [PubMed - indexed for MEDLINE]

☐ 14: Int J Cancer 2002 Apr 1;98(4):624-9 Related Articles, Links

Inhibition of PC-3 human prostate cancers by analogs of growth hormone-releasing hormone (GH-RH) endowed with vasoactive intestinal peptide (VIP) antagonistic activity.

Plonowski A, Varga JL, Schally AV, Krupa M, Groot K, Halmos G.

Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, New Orleans, LA 70112-1262, USA.

Vasoactive intestinal peptide (VIP) stimulates the proliferation and invasiveness of malignant prostatic cells. Receptors for VIP and the closely related growth hormone-releasing hormone (GH-RH) show considerable homology and are found in prostatic and other carcinomas. Among various analogs of GH-RH synthesized, JV-1-52 is a non-selective VIP/GH-RH antagonist, whereas JV-1-53 is a VIP antagonist devoid of GH-RH antagonistic effect. In our study, nude mice bearing PC-3 human androgen-independent prostate carcinomas were treated with JV-1-52 or JV-1-53 (20 microg/day, s.c.) for 28 days. Both antagonists produced a similar reduction in tumor volume (62-67%, $p < 0.01$) and tumor weight (59-62%; $p < 0.05$) vs. controls and extended tumor doubling-time from 9.1 to about 16 days ($p < 0.05$). To investigate the mechanisms involved, in another study we compared the effects of JV-1-53 with those of somatostatin analog RC-160. VIP antagonist JV-1-53 reduced tumor weight by 67% ($p < 0.01$) and suppressed the expression of mRNA for c-fos and c-jun oncogenes by about 34% ($p < 0.05$), without affecting serum levels of insulin-like growth factor-I (IGF-I). In contrast, RC-160 (50 microg/day) reduced serum IGF-I by 19% ($p < 0.05$), but did not significantly decrease tumor weight. mRNA for VIP and high affinity receptors

for VIP were detected on PC-3 tumors. Our results suggest that VIP/GH-RH antagonists can inhibit the growth of androgen-independent prostate cancer by abrogating the autocrine/paracrine mitogenic stimuli of VIP. The ability of GH-RH antagonists to block tumoral VIP receptors, in addition to GH-RH receptors, could be potentially beneficial for prostate cancer therapy. Copyright 2002 Wiley-Liss, Inc.

PMID: 11920625 [PubMed - indexed for MEDLINE]

☐ 15: Prostate 2000 Jul 1;44(2):172-80 Related Articles, Links

Potentiation of the inhibitory effect of growth hormone-releasing hormone antagonists on PC-3 human prostate cancer by bombesin antagonists indicative of interference with both IGF and EGF pathways.

Plonowski A, Schally AV, Varga JL, Rekasi Z, Hebert F, Halmos G, Groot K.

Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, New Orleans, Louisiana, USA.

BACKGROUND: In view of the involvement of various neuropeptides and growth factors in the progression of androgen-independent prostate cancer, we investigated the effects of antagonists of growth hormone-releasing hormone (GHRH) alone or in combination with an antagonist of bombesin/gastrin-releasing peptide (BN/GRP) on PC-3 human prostate cancers. METHODS: Nude mice implanted with PC-3 tumors received GHRH antagonists MZ-5-156 or JV-1-38, each at 20 microgram/day s.c. In experiment 2, treatment consisted of daily injections of JV-1-38 (20 microgram), BN/GRP antagonist RC-3940-II (10 microgram), or a combination of JV-1-38 and RC-3940-II. Serum IGF-I levels, expression of mRNA for IGF-II, and characteristics of BN/GRP and EGF receptors in tumor tissue were investigated. RESULTS: JV-1-38 induced a greater inhibition of tumor growth and suppression of IGF-II mRNA than MZ-5-156, both compounds causing a similar decrease in serum IGF-I. In experiment 2, JV-1-38 and RC-3940-II produced a comparable reduction in tumor volume (65% and 61%, respectively), but a combination of both antagonists augmented tumor inhibition to 75%. Combined treatment with JV-1-38 and RC-3940-II also led to a greater suppression of IGF-II mRNA (92%), as compared with JV-1-38 (72%) or RC-3940-II (77%). Serum IGF-I concentration was lowered only in mice treated with JV-1-38, while the downregulation of BN/GRP and EGF receptors was specific for groups receiving RC-3940-II. CONCLUSIONS: The inhibitory effects of GHRH antagonists on PC-3 human androgen-independent prostate cancer can be potentiated by concomitant use of BN/GRP antagonists. The combination of both types of analogs apparently interferes with both IGF and

bombesin/EGF pathways, and might be clinically useful for the management of androgen-independent prostate cancer. Copyright 2000 Wiley-Liss, Inc.

PMID: 10881027 [PubMed - indexed for MEDLINE]

☐ 16: Regul Pept 1998 Oct 16;77(1-3):185-92 Related Articles, Links

Luteinizing hormone-releasing hormone (LH-RH) antagonist Cetrorelix inhibits growth of DU-145 human androgen-independent prostate carcinoma in nude mice and suppresses the levels and mRNA expression of IGF-II in tumors.

Lamharzi N, Schally AV, Koppan M.

Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, New Orleans, LA 70146, USA.

In previous studies, we showed that LH-RH antagonist Cetrorelix inhibits the growth of DU-145 and PC-3 human androgen-independent prostate cancers in nude mice. To investigate the mechanisms involved, we treated male nude mice bearing xenografts of DU-145 human androgen-independent prostate cancer with Cetrorelix at a dose of 100 microg/animal subcutaneously (s.c.) once a day. Tumor growth, serum and tumor levels of IGF-I and -II as well as the mRNA expression of IGF-I and -II in tumors were evaluated. After 8 weeks of treatment, final volume and weight of DU-145 tumors in mice treated with Cetrorelix were significantly decreased compared with controls and serum IGF-1 showed a significant reduction. Therapy with Cetrorelix also reduced by 84% the levels of IGF-II in DU-145 tumor tissue compared with controls, but did not affect the concentration of IGF-I. RT-PCR analyses revealed a high expression of mRNA for IGF-II, but not for IGF-I in DU-145 tumors. Treatment with Cetrorelix decreased the expression of IGF-II mRNA by 78% ($p < 0.01$) as compared with controls. Our study indicates that LH-RH antagonist Cetrorelix may inhibit the growth of DU- 145 human androgen-independent prostate cancers by decreasing the production and mRNA expression of IGF-II by the tumor tissue. This also suggests that LH-RH antagonist Cetrorelix could interfere with the signal transduction pathways involving IGF-II, leading to tumor growth inhibition.

PMID: 9809814 [PubMed - indexed for MEDLINE]

☐ 17: Br J Cancer 1997;75(11):1585-92 Related Articles, Links

Inhibition of in vivo proliferation of androgen-independent prostate cancers by an antagonist of growth hormone-releasing hormone.

Jungwirth A, Schally AV, Pinski J, Halmos G, Groot K, Armatis P, Vadillo-Buenfil M.

Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center and Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146, USA.

Tumour-inhibitory effects of a new antagonist of growth hormone-releasing hormone (GH-RH), MZ-4-71, were evaluated in nude mice bearing androgen-independent human prostate cancer cell lines DU-145 and PC-3 and in Copenhagen rats implanted with Dunning R-3327 AT-1 prostatic adenocarcinoma. After 6 weeks of therapy, the tumour volume in nude mice with DU-145 prostate cancers treated with 40 microg day(-1) MZ-4-71 was significantly decreased to 37 +/- 13 mm3 ($P < 0.01$) compared with controls that measured 194 +/- 35 mm3. A similar inhibition of tumour growth was obtained in nude mice bearing PC-3 cancers, in which the treatment with MZ-4-71 for 4 weeks diminished the tumour volume to 119 +/- 35 mm3 compared with 397 +/- 115 mm3 for control animals. Therapy with MZ-4-71 also significantly decreased weights of PC-3 and DU-145 tumours and increased tumour doubling time. Serum levels of GH and IGF-I were significantly decreased in animals treated with GH-RH antagonist. In PC-3 tumour tissue, the levels of IGF-I and IGF-II were reduced to non-detectable values after therapy with MZ-4-71. The growth of Dunning R-3327 AT-1 tumours in rats was also significantly inhibited after 3 weeks of treatment with 100 microg of MZ-4-71 day(-1) i.p. as shown by a reduction in tumour volume and weight (both P-values < 0.05). Specific high-affinity binding sites for IGF-I were found on the membranes of DU-145, PC-3 and Dunning R-3327 AT-1 tumours. Our results indicate that GH-RH antagonist MZ-4-71 suppresses growth of PC-3, DU-145 and Dunning AT-1 androgen-independent prostate cancers, through diminution of GH release and the resulting decrease in the secretion of hepatic IGF-I, or through mechanisms involving a lowering of tumour IGF-I levels and possibly an inhibition of tumour IGF-I and IGF-II production. GH-RH antagonists could be considered for further development for the therapy of prostate cancer, especially after the relapse.

PMID: 9184172 [PubMed - indexed for MEDLINE]

Oncology 2002;63(4):317-32 Related Articles, Links

The role of the IGF system in cancer: from basic to clinical studies and clinical applications.

Moschos SJ, Mantzoros CS.

Division of Endocrinology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Mass 02215, USA.

Insulin-like growth factors (IGFs) are important mediators of growth, development, and survival, are synthesized by almost any tissue in the body, and their action is modulated by a complex network of molecules, including binding proteins, proteases and receptors, which all comprise the IGF system. Evidence from in vitro and animal studies suggests that over expression of IGFs by cancer cells and/or the nearby stroma as well as the type IGF-I receptor by the cancer cells may play a significant role in establishing a transformed phenotype in an increasing number of malignancies. More specifically, IGFs may promote cell cycle progression and inhibition of apoptosis either by directly associating with other growth factors or indirectly by interacting with other molecular systems which have an established role in carcinogenesis and cancer promotion, such as the steroid hormones and integrins. In addition, a growing number of epidemiologic studies suggest that increased serum levels of IGFs and/or altered levels of their binding proteins are associated with increased risk for developing several malignancies. These data indicate that IGF dysregulation should now be considered as an important independent factor for cancer risk, and a potential target for novel antineoplastic therapies and/or preventative strategies in high-risk groups. Copyright 2002 S. Karger AG, Basel

Curr Cancer Drug Targets 2002 Sep;2(3):191-207 Related Articles, Links

Insulin-like growth factor receptor-1 as an anti-cancer target: blocking transformation and inducing apoptosis.

Wang Y, Sun Y.

Cancer Molecular Sciences, Pfizer Global Research and Development, Ann Arbor Laboratories, 2800 Plymouth Road, Ann Arbor, MI 48105, USA.

IGF-IR (Insulin-like growth factor receptor 1) is a tetrameric glycoprotein composed of two alpha and two beta subunits. The alpha subunit localizes extracellularly for ligand binding, whereas the beta subunit consists of transmembrane chains and a cytoplasmic tyrosine kinase domain for enzymatic activity. IGF-IR ligands, IGF-I and IGF-II, are mitogens and survival factors for many cancer cells. Binding of ligands to the IGF-IR initiates a cascade of events leading to

activation of signal transduction pathways, mainly MAPK and PI-3K pathways, to stimulate proliferation/mitogenesis, to induce neoplastic transformation, to inhibit apoptosis, and to promote angiogenesis and metastasis. It has been shown that the presence of IGF-IR was required for transformation induced by many oncogenes and over-expression or constitutive activation of IGF-IR gave rise to transformed phenotypes. Significantly, over-expression of IGF-IR was observed in multiple human cancers including carcinomas of breast, lung, colon, and prostate. Patients with IGF-IR positive cancers had a worse prognosis in some cases. Furthermore, down-regulation or functional inactivation of IGF-IR sensitized tumor cells to apoptosis and reversed tumor cell phenotype. Thus, IGF-IR appears to be a promising cancer target. Indeed, a variety of approaches aimed at targeting IGF-IR have been utilized to prove the concept, or are being developed for potential anticancer therapies. These include targeting functional IGF-IR on cell surface, targeting ligand/receptor interaction, targeting receptor expression and functions, and targeting receptor kinase activity. Cancer patients could eventually benefit from the development of these specific IGF-IR antagonists.

Lancet Oncol 2002 May;3(5):298-302 Related Articles, Links

Insulin-like growth factors and cancer.

Furstenberger G, Senn HJ.

Center for Tumour Detection and Prevention, St Gallen, Switzerland.
gfuerstenberger@sg.zetup.ch

Interest in insulin-like growth factors (IGFs) and their effect on carcinogenesis has increased recently because high serum concentrations of IGF1 are associated with an increased risk of breast, prostate, colorectal, and lung cancers. Physiologically, IGF1 is the major mediator of the effects of the growth hormone; it thus has a strong influence on cell proliferation and differentiation and is a potent inhibitor of apoptosis. The action of IGF1 is predominantly mediated through the IGF1 receptor (IGF1R). IGF1R is involved in several oncogenic transformation processes. The availability of unbound IGF1 for interaction with IGF1R is modulated by IGF-binding proteins (IGFBP1-6). IGFBPs, especially IGFBP3, have independent effects on cell growth, for example, IGFBP3 has proapoptotic activities both dependent on and independent of p53

Mol Genet Metab 2000 Sep-Oct;71(1-2):315-20 Related Articles, Links

Regulation of the insulin-like growth factor-I receptor gene by oncogenes and antioncogenes: implications in human cancer.

Werner H, Shalita-Chesner M, Abramovitch S, Idelman G, Shaharabani-

Gargir L, Glaser T.

Department of Clinical Biochemistry, Tel Aviv University, Tel Aviv, 69978, Israel. hwerner@post.tau.ac.il

The insulin-like growth factor-I receptor (IGF-I-R) has a central role in normal cellular proliferation as well as in transformation processes. Transcription of the IGF-I receptor gene is controlled by a number of tumor suppressors, including WT1, p53, and BRCA1. It has been demonstrated that, in their wild-type form, these transcription factors can suppress the activity of the IGF-I-R promoter, with ensuing reduction in the levels of cell-surface IGF binding. On the other hand, a number of oncogenes, including mutant p53 and c-myb, and the fusion protein EWS-WT1 significantly stimulate promoter activity. Interactions between stimulatory and inhibitory transcription factors may determine the level of expression of the IGF-I-R gene and, consequently, the proliferative status of the cell.

J Natl Cancer Inst 2000 Sep 20;92(18):1472-89 Related Articles, Links

Role of the insulin-like growth factor family in cancer development and progression.

Yu H, Rohan T.

Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport 71130-3932, USA. hyu@lsumc.edu

The insulin-like growth factors (IGFs) are mitogens that play a pivotal role in regulating cell proliferation, differentiation, and apoptosis. The effects of IGFs are mediated through the IGF-I receptor, which is also involved in cell transformation induced by tumor virus proteins and oncogene products. Six IGF-binding proteins (IGFBPs) can inhibit or enhance the actions of IGFs. These opposing effects are determined by the structures of the binding proteins. The effects of IGFBPs on IGFs are regulated in part by IGFBP proteases. Laboratory studies have shown that IGFs exert strong mitogenic and antiapoptotic actions on various cancer cells. IGFs also act synergistically with other mitogenic growth factors and steroids and antagonize the effect of antiproliferative molecules on cancer growth. The role of IGFs in cancer is supported by epidemiologic studies, which have found that high levels of circulating IGF-I and low levels of IGFBP-3 are associated with increased risk of several common cancers, including those of the prostate, breast, colorectum, and lung. Evidence further suggests that certain lifestyles, such as one involving a high-energy diet, may increase IGF-I levels, a finding that is supported by animal experiments indicating that IGFs may abolish the inhibitory effect of energy restriction on cancer growth. Further investigation of the role of IGFs in linking high energy intake, increased cell proliferation, suppression of

apoptosis, and increased cancer risk may provide new insights into the etiology of cancer and lead to new strategies for cancer prevention

Eur J Cancer 2000 Jun;36(10):1224-8 Related Articles, Links

Insulin-like growth factor physiology and cancer risk.

Pollak M.

Cancer Prevention Research Unit, Lady Davis Research Institute of the Jewish General Hospital and McGill University, Montreal, H3T 1E2, Quebec, Canada. md49@musica.mcgill.ca

In the past few years, both laboratory investigations and population studies have provided strong circumstantial evidence that insulin-like growth factor (IGF) physiology influences cancer risk. In contrast to the influence of germ line mutations that are rare but are associated with very high risks, the impact of inter-individual variability in IGF physiology on risk appears to be modest but to effect a relatively high percentage of the population. Although this field of investigation is young, attention is already being given to the possibility that it may be relevant to clinical assessment of risk and/or to the identification of novel prevention strategies and intermediate endpoints. This review summarises key results in this field and provides a hypothesis concerning the mechanism by which IGF physiology influences risk of common epithelial cancers including those of breast, prostate, lung and colón.

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1: Anticancer Res 1994 Mar-Apr;14(2A):427-31 Related Articles, Books, LinkOut

The effect of droloxifene on the insulin-like growth factor-I-stimulated growth of breast cancer cells.

Kawamura I, Lacey E, Mizota T, Tsujimoto S, Nishigaki F, Manda T, Shimomura K.

Pharmacological Res. Lab., Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

Insulin-like growth factor-I (IGF-I) is an important mitogen in breast cancer. We studied here the effects of a new antiestrogen drug, droloxifene (DROL, (E)-alpha-[p-[2-(dimethylamino) ethoxy]-phenyl]-alpha'-ethyl-3-stilbenol) and tamoxifen (TAM) on the IGF-I-stimulated growth of estrogen receptor (ER) positive breast cancer cells, MCF-7 and their mechanism of action. IGF-I secretion from MCF-7 was increased by the addition of estrogen. Externally added IGF-I stimulated the growth of MCF-7 but not ER negative breast cancer cells, MDA-MB-231. DROL and TAM inhibited the IGF-I-stimulated growth of MCF-7. A 2 hr treatment with both drugs did not block IGF-I binding to the receptors in MCF-7. However, a 4 day treatment with DROL decreased the number of IGF-I receptors without altering the binding affinity in MCF-7. These results suggest that DROL can exert its antitumor activity against ER positive breast cancer not only by blocking the E2 binding to the ER, but also by counteracting the mitogenic effect of IGF-I.

PMID: 8017842 [PubMed - indexed for MEDLINE]

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Insulin-like growth factors in breast cancer.

Helle SI, Lonning PE.

Department of Oncology, Haukeland University Hospital, Bergen, Norway.

Insulin-like growth factor (IGF)-I is one of the most potent mitogens to many breast cancer cell lines in vitro. Effective growth inhibition in vitro may be achieved by antibodies to the type I IGF receptor (IGF-IR) or by using antisense strategies. Most human breast cancers express IGF-IR in vivo. Thus, different therapeutic strategies aimed at inhibiting ligand stimulation of the IGF-IR may be an attractive treatment option against breast cancer. Several drugs commonly used in breast cancer influence the IGF system both in vitro and in vivo. While antioestrogens such as tamoxifen and droloxifene reduce the expression of IGF-IR in vitro and suppress plasma levels of IGF-I but elevate IGF-binding protein-1 in vivo, megestrol acetate may reduce the delivery of IGFs to the tissues by inhibition of IGFBP-3 protease activity.

Publication Types:
- Review
- Review, Tutorial

PMID: 9142960 [PubMed - indexed for MEDLINE]

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Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells.

Lee AV, Weng CN, Jackson JG, Yee D.

Department of Medicine, University of Texas Health Science Center at San Antonio 78284-7884, USA.

Estrogen and IGF-I are potent mitogens for most breast cancer cell lines, and although their signaling pathways contrast, there is considerable interaction between them. Recent evidence indicating that IGF-I can alter estrogen receptor (ER) action led us to investigate whether an inhibitor of IGF-I action. IGF-binding protein-1 (IGFBP-1), could affect transcriptional activation of ER. First, we confirmed that tamoxifen (TAM) could inhibit IGF-I-mediated proliferation of MCF-7 cells. Although TAM can increase IGFBP-3 expression in MCF-7 cells, and this binding protein has been shown to be able to inhibit IGF action, TAM had no effect on IGF-I-stimulated tyrosine phosphorylation of IGF-I receptor or the downstream signaling molecule, insulin receptor substrate-1. Therefore, to confirm that IGF-I was affecting transcriptional activation of ER, we utilized a gene reporter assay using a single consensus estrogen response element (ERE-tk-luc) upstream of luciferase. As expected, estradiol (E2; 1nM) increased transcriptional activation three- to fivefold from the ERE in three ER-positive breast cancer cell lines (MCF-7, ZR-75 and T47D). A 2.5-to 4-fold increase was also seen with IGF-I (5 nM). TAM (1 microM) effectively blocked activation by E2 and IGF-I, indicating disruption of ER-mediated transcription. As expected, human recombinant IGFBP-1 (80 nM) completely inhibited IGF-I-mediated activation of ER, however, IGFBP-1 also caused a significant decrease in E2-mediated activation. We also noticed that IGF-I increased the activity of all plasmids that we cotransfected including TATA-luc, SV40-luc and pGL Basic. This effect was post-transcriptional, as it was not affected by actinomycin D (2 micrograms/ml), while we were able to completely inhibit E2-mediated transcriptional activation of ERE-tk-luc. Unlike the complete inhibition of ER-mediated transcriptional activation by actinomycin D, IGF-I-mediated transactivation was reduced by only 50%, indicating that the activation by IGF-I represented both transcriptional and post-transcriptional effects. This study confirmed that IGF-I can cause transcriptional activation of

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Inhibition of insulin- and insulin-like growth factor-I-stimulated growth of human breast cancer cells by 1,25-dihydroxyvitamin D3 and the vitamin D3 analogue EB1089.

Vink-van Wijngaarden T, Pols HA, Buurman CJ, Birkenhager JC, van Leeuwen JP.

Department of Internal Medicine III, Erasmus University, Rotterdam, Netherlands.

1,25 Dihydroxyvitamin D3 (1,25-(OH)2D3) and a number of synthetic vitamin D3 analogues with low calcaemic activity, have been shown to inhibit breast cancer cell growth in vitro as well as in vivo. The purpose of the present study was to investigate a possible interaction of 1,25-(OH)2D3 and the vitamin D3 analogue EB1089 with the insulin-IGF-I regulatory system. The oestrogen receptor-positive MCF-7 human breast cancer cells used in this study are able to grow autonomously and their growth is stimulated by insulin. In order to avoid interference of IGF-binding proteins (IGF-BPs), we used an analogue of IGF-I, long R3 IGF-I, which stimulated MCF-7 cell growth similar to insulin. The growth stimulation by insulin and by long R3 IGF-I was completely inhibited by 1,25-(OH)2D3 and EB1089. Autonomous growth was also inhibited by 1,25-(OH)2D3 and EB1089. The analogue EB1089 was active at 50 times lower concentrations than 1,25-(OH)2D3. It was shown that growth inhibition was not achieved through downregulation of insulin and IGF-I binding after 48 h. Paradoxically, after prolonged treatment (8 days), an upregulation of insulin and IGF-I binding was observed. Two possible intracellular mediators of the insulin-IGF mitogenic signal are C-FOS and mitogen-activated protein (MAP) kinase. Insulin-induced C-FOS mRNA was inhibited by 1,25-(OH)2D3, suggesting that it could be involved in the growth inhibition by 1,25-(OH)2D3. MAP kinase activation appeared not to be involved in growth stimulation by both insulin and IGF-I. Together, the present study demonstrates that vitamin D3 compounds can block the mitogenic activity of insulin and IGF-I, which may contribute to their tumour suppressive activity observed in vivo.

PMID: 9081364 [PubMed - indexed for MEDLINE]

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IGF-I physiology and breast cancer.

Pollak M.

Department of Medicine, McGill University, Montreal, Quebec, Canada.

Recent studies imply that IGF-I levels vary greatly between normal women, and that premenopausal breast cancer risk is increased among women with higher IGF-I levels. It is known that tamoxifen lowers IGF-I levels, but further research is needed to determine whether antiestrogens will be of particular value in risk reduction for women with high IGF-I levels, and also to determine if IGF-I levels can indeed be used as an intermediate endpoint in risk reduction interventions. With respect to adjuvant therapy, we currently have convincing data that antiestrogens have moderate IGF-I lowering actions, but it remains unclear to what extent these contribute to the therapeutic effect of these compounds. Ongoing trials are addressing this question, as well as the hypothesis that interventions that increase IGF-I suppression will be associated with reduced relapse rates.

Publication Types:
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- Lancet. 1998 May 9;351(9113):1373-5.

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Circulating concentrations of insulin-like growth factor-I and risk of breast cancer.

Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FE, Pollak M.

Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA.

BACKGROUND: Insulin-like growth factor (IGF)-I, a mitogenic and antiapoptotic peptide, can affect the proliferation of breast epithelial cells, and is thought to have a role in breast cancer. We hypothesised that high circulating IGF-I concentrations would be associated with an increased risk of breast cancer. METHODS: We carried out a nested case-control study within the prospective Nurses' Health Study cohort. Plasma concentrations of IGF-I and IGF binding protein 3 (IGFBP-3) were measured in blood samples collected in 1989-90. We identified 397 women who had a diagnosis of breast cancer after this date and 620 age-matched controls. IGF-I concentrations were compared by logistic regression with adjustment for other breast-cancer risk factors. FINDINGS: There was no association between IGF-I concentrations and breast-cancer risk among the whole study group. In postmenopausal women there was no association between IGF-I concentrations and breast-cancer risk (top vs bottom quintile of IGF-I, relative risk 0.85 [95% CI 0.53-1.39]). The relative risk of breast cancer among premenopausal women by IGF-I concentration (top vs bottom tertile) was 2.33 (1.06-5.16; p for trend 0.08). Among premenopausal women less than 50 years old at the time of blood collection, the relative risk was 4.58 (1.75-12.0; p for trend 0.02). After further adjustment for plasma IGFBP-3 concentrations these relative risks were 2.88 and 7.28, respectively. INTERPRETATION: A positive relation between circulating IGF-I concentration and risk of breast cancer was found among premenopausal but

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mRNA expression of components of the insulin-like growth factor system in breast cancer cell lines, tissues, and metastatic breast cancer cells.

Gebauer G, Jager W, Lang N.

Department of Obstetrics and Gynaecology, University of Erlangen-Nuremberg, Erlangen, Germany. gerhard.gebauer@rzmail.uni-erlangen.de

IGF-1 and 2 are thought to be important growth factors for breast cancer. However, gene expression of IGFs or IGF receptors in breast cancer tissues, and especially in metastatic breast cancer cells, is not well known. Expression of mRNA encoding for IGF-1, IGF-2, IGF-receptor 1 and 2, IGF binding proteins- 1 to -6, insulin receptor and insulin was determined in the NIH MCF-7 breast cancer cell line, in specimens from breast cancer tissues, and in 6 primary breast cancer cell cultures obtained from metastatic breast cancer, using rT-PCR technique. Specific mRNA sequences encoding for IGF-receptor 1 and 2, IGFBP-2, -4 and insulin receptor were identified in all cell cultures and most of the tissue specimens. Though in most of the tissues additional expression of IGF-1 and IGF-2 was detected, there was no mRNA encoding for these proteins in MCF-7 cell cultures as well as in the primary cell cultures of metastatic breast cancers. In none of our specimens mRNA encoding for IGFBP-1, -3, -5, -6 and insulin was detectable. IGF-receptor expression in cancer tissues and metastatic breast cancer cells supports the hypothesis that IGFs increase tumor cell proliferation in vivo. Expression of IGF-1 and IGF-2 in tumor tissues but not in cancer cell cultures indicates an IGF expression located predominantly in stromal parts of cancer tissues.

PMID: 9615787 [PubMed - indexed for MEDLINE]

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1: Eur J Cancer Prev 1997 Aug;6(4):330-40 Related Articles, Books, LinkOut

Reduced testosterone, 17 beta-oestradiol and sexual hormone binding globulin, and increased insulin-like growth factor-1 concentrations, in healthy nulligravid women aged 19-25 years who were first and/or second degree relatives to breast cancer patients.

Jernstrom HC, Olsson H, Borg A.

Department of Oncology, University Hospital, Lund, Sweden.

Differences in hormonal and constitutional parameters between women with at least one first and/or second degree relative with breast cancer (RBC) and women without such affected relatives were studied in a group of healthy, nulligravid women aged 19-25 years. Present oral contraceptive (OC) users were analysed separately. In women not presently exposed to OCs we found significant correlations between RBC and reduced concentrations of testosterone during both the follicular ($P < 0.001$) and luteal menstrual cycle phases ($P = 0.016$). 17 beta-oestradiol was also significantly negatively correlated with RBC in the follicular ($P = 0.044$) and in the luteal phase ($P = 0.027$). RBC was significantly correlated with a lower waist/hip ratio ($P = 0.044$) compared with women without such a history. In multivariate analyses, the results for testosterone but not 17 beta-oestradiol remained significant. In these analyses high IGF-1 ($P = 0.05$) in the follicular phase and low sexual hormone-binding globulin (SHBG) ($P = 0.04$) in the luteal phase were also related to RBC. Including all 66 women in a multivariate model that analysed the specific effects from OCs and RBC on plasma testosterone showed that plasma testosterone was significantly lower among present OC users ($P = 0.004$) and in women with RBC ($P = 0.005$) during cycle days 5-10, with a significant positive two-way interaction between present OC use and RBC ($P = 0.007$). During cycle days 18-23 plasma testosterone showed a significant negative relationship with present OC use ($P < 0.001$) and RBC ($P = 0.016$) no significant interaction was seen during cycle days 18-23. Factors not significantly related to RBC were height, weight, breast size, age at menarche, p-progesterone and p-prolactin. It is concluded that a family history of breast cancer significantly lowered plasma testosterone concentrations in both cycle phases among healthy, nulligravid women compared with women without such history.

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1: Oncology 1999 Nov;57(4):330-40 Related Articles, Books, LinkOut

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Plasma insulin-like growth factor-I and serum IGF-binding protein 3 can be associated with the progression of breast cancer, and predict the risk of recurrence and the probability of survival in African-American and Hispanic women.

Vadgama JV, Wu Y, Datta G, Khan H, Chillar R.

Department of Medicine, Divisions of Laboratory Research and Development and Hematology/Oncology, Charles R. Drew University of Medicine and Science, and UCLA School of Medicine, Los Angeles, CA 90059, USA.

In vitro studies have shown that insulin-like growth factor (IGF) is a mitogen for breast cancer cells. However, the associations of plasma IGF-I with tumor histopathology in high-risk groups need further investigation. We hypothesize that plasma IGF-I and serum IGFBP3 concentrations in breast cancer patients may provide useful information on the progression of their disease, and determine the probability of recurrence and survival. We have carried out a retrospective study on 130 minority breast cancer patients. Plasma IGF-I and serum IGFBP3 were correlated with tumor histopathology, menopausal status, treatment modality, recurrence rates, and probability of survival. Plasma IGF-I and serum IGFBP3 were measured by radioimmunoassay. Our studies show that breast cancer patients have elevated plasma IGF-I and serum IGFBP3 levels. In addition we observed the following: IGF-I did not correlate with age and nodal stage. IGF-I and IGFBP3 increased with tumor size (T4). IGF-I did not correlate with estrogen receptor status, but did increase in progesterone-receptor-positive patients. IGF-I levels were higher in premenopausal patients and in women with cancer recurrence. Tamoxifen reduced IGF-I levels significantly and reduced the risk of recurrence. The survival probability was greater in patients with plasma IGF-I levels <120 ng/ml. In conclusion, lowering of plasma IGF-I may offer the following benefits: (a) reduce the risk of developing breast cancer in high-risk groups; (b) slow the progression of breast cancer in patients at early stages of cancer; (c) lower the risk of recurrence, and (d) increase the probability of survival. Copyright Copyright 1999 S. Karger AG, Basel

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1: Eur J Cancer 1999 Nov;35(12):1717-23 Related Articles, Books, LinkOut

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Vitamin D analogues suppress IGF-I signalling and promote apoptosis in breast cancer cells.

Xie SP, Piriamov G, Colston KW.

Department of Oncology, Gastroenterology, Endocrinology and Metabolism, St George's Hospital Medical School, London, U.K.

Survival factors are known to promote cell viability, and factor deprivation can be a potent apoptotic signal. Insulin-like growth factors are potent mitogens and inhibitors of apoptosis for many normal and neoplastic cells with insulin-like growth factor-I (IGF-I) being the most effective in many breast cancer cell lines. 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) and its analogues inhibit IGF-I-stimulated growth of MCF-7 human breast cancer cells. The aim of this study was to determine the relationship between inhibition of IGF-I responsiveness and induction of apoptosis by vitamin D analogues in breast cancer cells. Vitamin D analogues EB1089 and CB1093 inhibited autonomous and IGF-I-stimulated growth of MCF-7 and T47D cells and autonomous growth of IGF-I-insensitive Hs578T cells. In MCF-7 cells, IGF-I alone (4 nM) protected against apoptosis mediated by serum deprivation. Co-treatment with vitamin D analogues prevented the anti-apoptotic effects of IGF-I. In T47D cells, IGF-I treatment provided only partial protection against apoptosis induced by serum deprivation and co-incubation of serum-deprived cells with 100 nM CB1093 and IGF-I abrogated this partial protection. In Hs578T cells, addition of IGF-I did not prevent apoptosis induced by serum deprivation. However, treatment with CB1093 attenuated the protective effect of the serum in these cells. Our findings suggest that vitamin D analogues inhibit IGF-I signalling pathways to promote apoptosis in breast cancer cells.

PMID: 10674019 [PubMed - indexed for MEDLINE]

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Insulin-like growth factor-I and human lung fibroblast-derived insulin-like growth factor-I stimulate the proliferation of human lung carcinoma cells in vitro.

Ankrapp DP, Bevan DR.

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg 24061.

The concentration of insulin-like growth factor I (IGF-I) in tissue taken from human non-small cell lung carcinomas (non-SCLC) is 1.4- to 7-fold higher than in the surrounding normal lung tissue, and thus, IGF-I may be involved in the growth of non-SCLC. We report here that non-SCLC cell lines (A549, A427, SK-LU-1) expressed the IGF-I receptor protein, and IGF-I stimulated the proliferation of low-density plated (2000 cells/cm2 growth area) carcinoma cells by 1.6- to 3-fold above control after a 4-day incubation period under serum-free conditions (A549, A427) or in the presence of 0.25% serum (SK-LU-1). Immunoblot data indicated that IGF-I was not secreted by the lung carcinoma cells; however, IGF-I-like proteins were present in the serum-free medium conditioned by human adult lung fibroblasts (CCD-19Lu). The secretion of the immunoreactive IGF-I-like protein was dependent on the passage level of the fibroblasts. At least one of the IGF-I-like factors promoted the serum-free growth of A549 cells (2-fold increase in cell number over control after 4 days) and stimulated a 3-fold increase in the tyrosine kinase activity of detergent-solubilized IGF-I receptors from A549 cells. Both stimulatory effects were neutralized by an anti-IGF-I antibody, suggesting that the fibroblast-derived factor mediated its activity via the IGF-I receptor. Our data indicate that lung fibroblast-derived IGF-I may stimulate the growth of non-SCLC in vivo.

PMID: 8391925 [PubMed - indexed for MEDLINE]

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Blood levels of IGF-I in non-small cell lung cancer: relation to clinical data.

Tisi E, Lissoni P, Rovelli F, Mandelli D, Barni S, Tancini G.

Division of Thoracic Surgery, San Gerardo Hospital, Monza, Italy.

Recent observations have demonstrated that somatomedins, mainly insulin-like growth factor-I (IGF-I), are growth factors for non-small cell lung cancer (NSCLC). On the basis of this evidence, a study was started to evaluate serum levels of IGF-I in a group of untreated NSCLC patients. The study included 46 patients, 25 of whom had an operable tumor, while the other 21 showed distant organ metastases. IGF-I and GH serum levels were measured by RIA in each patient; moreover, in operable patients, hormonal detections were made either before, or 7 days after surgery. The control group comprised 38 age-matched healthy subjects. Mean serum levels of IGF-I were significantly higher in cancer patients with respect to controls, while no difference was seen in mean GH values. Moreover, patients with metastases showed significantly higher levels of IGF-I than the patients without. Within the operable group, patients with lung adenocarcinoma had higher levels of IGF-I than those with epidermoid cell carcinoma, but this difference was not significant. Finally, no significant difference in IGF-I mean values was seen before and after surgical removal of tumors. This preliminary study shows that NSCLC patients may present abnormally high levels of IGF-I. Because of the stimulating role of IGF-I on NSCLC growth, this evidence could play a role in the clinical course of neoplastic lung disease.

PMID: 1653807 [PubMed - indexed for MEDLINE]

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☐ 1: Eur J Cancer Clin Oncol 1988 Jul;24(7):1241-2 Related Articles, Books, LinkOut

Serum insulin-like growth factor-I levels in patients with small cell lung cancer.

Macaulay VM, Teale JD, Everard MJ, Joshi GP, Millar JL, Smith IE.

Publication Types:
- Letter

PMID: 2843377 [PubMed - indexed for MEDLINE]

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1: Br J Cancer 1988 Jan;57(1):91-3

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Somatomedin-C/insulin-like growth factor-I is a mitogen for human small cell lung cancer.

Macauly VM, Teale JD, Everard MJ, Joshi GP, Smith IE, Millar JL.

Department of Medicine, Royal Marsden Hospital, Sutton, Surrey, UK.

PMID: 2831929 [PubMed - indexed for MEDLINE]

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☐ 1: Anticancer Res 1999 Mar-Apr;19(2B):1397-9 Related Articles, Books, LinkOut

Decreased serum levels of insulin-like growth factor (IGF)-I in patients with lung cancer: temporal relationship with growth hormone (GH) levels.

Mazzoccoli G, Giuliani A, Bianco G, De Cata A, Balzanelli M, Carella AM, La Viola M, Tarquini R.

Department of Internal Medicine, Regional General Hospital Casa Sollievo della Sofferenza, Italy.

AIMS AND BACKGROUND: Several studies have evidenced that IGF-1 may play a role in the growth regulation of many cancer cell lines, and recently GH and IGF-1 have been recognized as stimulators of lymphopoiesis and immune function. We investigated whether there are differences among health- old people and old people suffering from lung cancer at different stages of disease in the 24-hour secretory profiles of GH und IGF-1. METHODS: The study was carried out on seven healthy volunteers (mean age +/- s.e. 68.8 + 1.92), seven patients with I and II stage lung cancer (mean age +/- s.e. 67.2 +/- 0.80) and seven patients with III and IV stage lung cancer (mean age +/- s.e. 69.5 +/- 2.26). GH and IGF-1 serum levels were measured on blood samples collected every four hours for 24 hours; the area under the curve (AUC) and the presence of circadian rhythmicity were evaluated. RESULTS: A normal circadian rhythmicity was recognizable only for GH secretion in healthy subjects. A progressive increase of GH serum levels and a steady decrease of IGF-1 serum levels were observed in cancer patients in relation to advancing stage of neoplastic disease. CONCLUSIONS: Lung cancer is associated with an altered regulation of GH-IGF-1 system, that might play a role in the clinical course of neoplastic disease.

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Anti-insulin-like growth factor-I activity of a novel polysulphonated distamycin A derivative in human lung cancer cell lines.

de Cupis A, Ciomei M, Pirani P, Ferrera A, Ardizzoni A, Favoni RE.

Department of Preclinical Oncology, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

1. The purpose of this study was to investigate the antiproliferative effect and the modulation of the mitogenic insulin-like growth factor-I (IGF-I) system by FCE 26644 and FCE 27784, two polyanionic sulphonated distamycin A derivative compounds, on two human non-small cell lung cancer (N-SCLC) cell lines. 2. For cell growth studies the colorimetric MTT and the thymidine incorporation assays were performed; the presence of IGF-I and IGF-binding proteins in conditioned media was revealed by radioimmunoassay and Western ligand blot, respectively. Variations at the IGF-I-receptor level were tested by binding studies on cell monolayers. 3. A significant concentration- and time-dependent cytostatic activity of FCE 26644 (IC50 approximately 200 micrograms ml-1 at 72 h) compared to its analogue FCE 27784 (IC50 > 800 micrograms ml-1) was observed in both cell lines studied. The IGF-I-stimulated proliferation of the IGF-I-responsive A549 cell line was abolished by 24 h of FCE 26644 treatment whereas FCE 27784 was inactive. FCE 26644 increased (4 to 6 fold) the secretion of IGF-I-like material and reduced the IGF-I binding (IC50 > 100 micrograms ml-1) in both A549 and Ca-Lu-1 cell lines. FCE 26644 (100 micrograms ml-1) did not affect the KD (approximately 0.5 nM) but reduced the Bmax and the number of receptor sites (50%). 4. Our findings demonstrate that the ability to down-regulate the cell proliferation of N-SCLC cell lines, shown by FCE 26644, depends at least partially, on interference with the "IGF-I mitogenic system'.

PMID: 9031761 [PubMed - indexed for MEDLINE]

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Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis.

Yu H, Spitz MR, Mistry J, Gu J, Hong WK, Wu X.

Section of Cancer Prevention and Control, Feist-Weiller Cancer Center, Louisiana State University Medical Center, Shreveport, USA.

BACKGROUND: Insulin-like growth factors (IGFs), in particular IGF-I and IGF-II, strongly stimulate the proliferation of a variety of cancer cells, including those from lung cancer. To examine the possible causal role of IGFs in lung cancer development, we compared plasma levels of IGF-I, IGF-II, and an IGF-binding protein (IGFBP-3) in patients with newly diagnosed lung cancer and in control subjects. METHODS: From an ongoing hospital-based, case-control study, we selected 204 consecutive patients with histologically confirmed, primary lung cancer and 218 control subjects who were matched to the case patients by age, sex, race, and smoking status. IGF-I, IGF-II, and IGFBP-3 plasma levels were measured by enzyme-linked immunosorbent assay and then divided into quartiles, based on their distribution in the control subjects. Associations between the IGF variables and lung cancer risk were estimated by use of odds ratios (ORs). Reported P values are two-sided. RESULTS: IGF and IGFBP-3 levels were positively correlated (all r>.27; all P<.001). High plasma levels of IGF-I were associated with an increased risk of lung cancer (OR = 2.06; 95% confidence interval [CI] = 1.19-3.56; P = .01), and this association was dose dependent in both univariate and multivariate analyses. Plasma IGFBP-3 showed no association with lung cancer risk unless adjusted for IGF-I level; when both of these variables were analyzed together, high plasma levels of IGFBP-3 were associated with reduced risk of lung cancer (OR = 0.48; 95% CI = 0.25-0.92; P = .03). IGF-II was not associated with lung cancer risk. CONCLUSIONS: Plasma levels of IGF-I are higher and plasma levels of IGFBP-3 are lower in patients with lung cancer than in control subjects. If these findings can be confirmed in prospective studies, measuring levels of IGF-I and IGFBP-3 in blood may prove useful in assessing lung cancer risk.

PMID: 9923856 [PubMed - indexed for MEDLINE]

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Evidence for an increased somatomedin-C/insulin-like growth factor I content in primary human lung tumors.

Minuto F, Del Monte P, Barreca A, Fortini P, Cariola G, Catrambone G, Giordano G.

Immunoreactive somatomedin-C/insulin-like growth factor I (SM-C/IGF I) content was measured in human neoplastic lung tissue obtained from surgery on 10 patients (seven epidermoid carcinoma, three adenocarcinoma), and in normal lung tissue obtained from the same excised portion. SM-C/IGF I content in lung tumors was 615 +/- 123 (SE) milliunits/g of tissue (range, 214-1531), significantly higher (P less than 0.01) than normal tissue (234 +/- 51 milliunits/g of tissue; range, 37-537); in particular, every subject showed a clear-cut difference of SM-C/IGF I content between neoplastic and normal tissue (ratio, 3.41 +/- 0.69; range, 1.4-7.2). The results were essentially unchanged when data were expressed relative to hemoglobin or DNA tissue content. By contrast, in peripheral plasma SM-C/IGF I concentration was 0.51 +/- 0.17 units/ml, significantly lower (P less than 0.01) than in 59- to 70-yr-old control subjects (1.10 +/- 0.13 units/ml). In conclusion, the lung tumors studied, irrespective of their histological structure, contain more SM-C/IGF I than does normal tissue. Whether this is due to a primary in situ production of SM-C/IGF I or is secondary to overproduction of other inducers such as platelet derived growth factor-like peptides is yet to be clarified. The reduced circulating SM-C/IGF I concentration seems to be related more to the nutritional status of the patients.

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1: Growth Horm IGF Res 1998 Dec;8 (6):473-9

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Role of insulin-like growth factor-I (IGF-I) receptor, IGF-I, and IGF binding protein-2 in human colorectal cancers.

Mishra L, Bass B, Ooi BS, Sidawy A, Korman L.

Department of Medicine, Department of Veterans' Affairs Medical Center and Georgetown University Medical Center, Washington DC 20422, USA.

The identification of novel autocrine/paracrine signaling pathways and possible markers represents an important component in the understanding of tumor growth control. In this study, we assessed the potential role of insulin-like growth factor-I (IGF-I), the IGF-I receptor (IGF-IR) and IGF binding protein-2 (IGFBP-2) in human colorectal cancer. Initi studies demonstrating increased IGF-I binding and IGF-IR density in human colon cancer tissue revealed that a component of iodinated (3-[125-I]iodotyrosyl) IGF-I (125I-ICGF-I) binding was not attributable to IGF-IR. Binding studies and Western blot analysis suggested that this second component of 125I-IGF-I binding could be due to IGFBP-2. Further analysis by a specific solution hybridization/RNase protection assay for IGF-IR mRNA levels, IGFBP-2 mRNA levels and in situ hybridization for IGFBP-2 localization, was carried out in nine patients with colon cancer. IGF-IR mRNA levels by RNAse protection assays were unchanged, whereas IGFBP-2 mRNA levels were increased 4-8-fold in patients witl colon cancer compared to controls. Three patients with Duke: stage C disease had the highest levels of IGFBP-2 mRNA. In situ hybridization studies localized IGFBP-2 mRNA to malignant cells and not to the surrounding stromal cells, suggesting an autocrine role for IGFBP-2. The discrepancy

between increased IGF-I binding, IGF-IR density, IGFBP-2 mRNA and the minimal modulation of the IGF-IR mRNA implies post-transcriptional regulation of IGF-IRs. Our results suggest that IGFBP-2 may be implicated in colon cancer metastases and prognosis. Its usefulness as a potential tumc marker should be further investigated.

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1: Int J Cancer 1999 Sep 24;83(1):15-7 Related Articles, Books, LinkC

InterScience

IGF-I and IGF-II in relation to colorectal cancer.

Manousos O, Souglakos J, Bosetti C, Tzonou A, Chatzidakis V, Trichopoulos D, Adami HO, Mantzoros C.

Department of Hygiene and Epidemiology, University of Athens Medical School, Goudi, Athens, Greece.

Recent data suggest that the IGF system plays an important role in the pathogenesis of several forms of human cancer, and there is evidence that IGFs acting in an autocrine and paracrine manner may also affect colorectal cancer risk. We have conducted a case-control study on the island of Crete, Greece, to examine the potential relation between circulating IGF-I and -II and their major binding protein (IGF-BP3), on the one hand, and colorectal cancer, on the other. IGF-I, IGF II and IGF-BP3 were determined in the serum from 41 patients with colorectal cancer and 50 healthy controls; data were analyzed using unconditional multiple logistic regressior adjusting for age, gender, education, height and BMI, as well as mutually. Both IGF-I and IGF-II were positively, while IGF BP3 was inversely, associated with risk for colorectal cancer, though none of these relations reached statistical significance However, individuals with IGF-I and -II values in the upper 2 tertiles of the respective distributions had a significantly elevated odds ratio for colorectal cancer (OR = 5.2, 95% confidence interval 1.0-26.8) compared with those in the lower tertile in both distributions. Our results provide evidenc that high levels of circulating IGF-I and -II might be associated with colorectal cancer. Copyright 1999 Wiley-Liss, Inc.

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ELSEVIER SCIENCE FULL-TEXT ARTICLE

Characterization of an antibody that can detect an activated IGF-I receptor in human cancers.

Rubini M, D'Ambrosio C, Carturan S, Yumet G, Catalano E, Shan S, Huang Z, Criscuolo M, Pifferi M, Baserga R.

University of Ferrara, Via L. Borsari 46, Ferrara, 44100, Italy

The type 1 insulin-like growth factor receptor (IGF-IR) plays an important role in malignant transformation and in apoptosis. Its role in human cancer has now been firmly established. IGF-IR signaling occurs only when the receptor i activated by its ligands, which induce autophosphorylation of the receptor at several tyrosine residues. Although the IGF-II (phosphorylated or not) can be detected in human cancers with conventional antibodies, it would be desirable to obtain antibodies that can detect the IGF-IR only when activated by its ligands. We describe and characterize in this paper such a antibody and show that it can be used in sections of human cancers to detect an autophosphorylated IGF-IR. This antibody will be useful in detecting autocrine or paracrine influences on normal and tumor cells and could eventually be also useful in diagnostic and prognostic studies of human primary and metastatic cancer. Copyright 1999 Academic Press.

PMID: 10438568 [PubMed - indexed for MEDLINE]

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Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I.

Akagi Y, Liu W, Zebrowski B, Xie K, Ellis LM.

Department of Cell Biology, The University of Texas M.D. Anderson Cancer Center, Houston 77030, USA.

We investigated the role of insulin-like growth factor (IGF)-I and IGF-binding proteins (IGFBPs) in the regulation of vascular endothelial growth factor (VEGF) expression in color cancer cells and the mechanism by which this regulation occurs. HT29 human colon cancer cells were treated with IGF I for various time periods. VEGF mRNA expression increased within 2 h and peaked at 24 h. SW620 colon cancer cells exhibited a peak induction of VEGF mRNA 8 h after IGF-I treatment. IGF-I induction of VEGF was confirmed at the protein level. In experiments using transient transfection of VEGF promoter-reporter constructs into HT29 cells, IGF-I increased the activity of the VEGF promoter, and pretreatment of HT29 cells with dactinomycin abrogated the induction of VEGF mRNA by IGF-I. The half-life of VEGF mRN was not prolonged by treatment with IGF-I. Blocking the activity of IGFBP-4 did not significantly modulate the effect o IGF-I induction of VEGF mRNA in HT29 cells. Treating cells with des-(1-3)-IGF-I (an active derivative of IGF-I that does not bind to binding proteins) had effects on VEGF mRNA expression that were similar to those of IGF-I. These findings suggest that IGF-I regulates VEGF expression in human color cancer cells by induction of transcription of the VEGF gene. IGFBPs do not significantly affect IGF-I induction of VEGF.

PMID: 9731515 [PubMed - indexed for MEDLINE]

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InterScience

Insulin-like growth factor-I promotes multidrug resistance in MCLM colon cancer cells.

Guo YS, Jin GF, Houston CW, Thompson JC, Townsend CM Jr.

Department of Surgery, The University of Texas Medical Branch, Galveston 77555-0527, USA.

Insulin-like growth factor-I (IGF-I) is known as a potent mitogen for a variety of cell types, including colon cancer cell lines. The objective of this study was to determine the effect of IGF-I on cell death induced by cytotoxic agents actinomyci D (Act-D), lovastatin (LOV), and doxorubicin (DOX) in the MCLM mouse colon cancer cell line, and the mechanisms involved. Subconfluent monolayer MCLM cells were treated with IGF-I (25 ng/ml) for 12 h in serum-free media. Various concentrations of cytotoxic agents then were added to the - cells that were incubated continually at 37 degrees C for 24 h Cell survival was determined with the MTT (3-[4-5-dimenthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which assesses mitochondrial function in living cells. The mRNA expression for multidrug resistance gene-1 (mdr-1), c-H-ras, and manganese superoxide dismutase (MnSOD) in cells treated with IGF-I was examined by Northern blot or RNase protection assays. The levels of p-glycoprotein, a drug efflux pump encoded by the mdr-1 gene, were assessed by Western immunoblotting. Results demonstrated that 1) IGF-I significantly inhibited the cell death and apoptosis of MCLM cells treated with Act-D, LOV, or DOX; 2) IGF-I increased mRNA expression for mdr-1, c-H-ras, and MnSOD; 3) the p-glycoproteins in cells treated with IGF-I or stably transfected with c-H-ras were elevated when compared with control.

These results suggest that IGF-I protects MCLM cells against death induced by cytotoxic agents; this acquired drug resistance may be mediated by multiple mechanisms, including promoting expression of mdr-1, c-H-ras, and MnSOD; whereas, the p-glycoprotein level stimulated by IGF may result partly from the increase of c-H-ras in the cells.

PMID: 9525472 [PubMed - indexed for MEDLINE]

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Insulin-like growth factor binding proteins as mediators of IGF-I effects on colon cancer cell proliferation.

Michell NP, Dent S, Langman MJ, Eggo MC.

Department of Medicine, University of Birmingham, Queen Elizabeth Hospital, Edgbaston, UK.

Human colon cancer cell lines COLO205, HT29 and SW620 ar known to secrete insulin-like growth factor II (IGF-II) and its modulatory binding proteins (IGFBPs). We have characterise the sensitivity of these cell lines to exogenous IGF-I and hav examined the effects of their autocrine IGFBPs on these responses. Cells cultured in serum-free medium were treatec with 1-100 ng/ml IGF-I, or des(1,3)IGF-I, a truncated IGF-I with low affinity for IGFBPs. DNA synthesis was determined b 24 h incorporation of 3H-thymidine. Experiments were repeated in the presence of 24 h cell-conditioned media containing endogenous IGFBPs. In all 3 cell lines, cell-conditioned media reduced sensitivity to IGF-I but not to des (1,3)IGF-I suggesting that IGFBPs in the cell-conditioned media of colon cells inhibit IGF-I action. IGFBPs in the cell layer and 24 h cell-conditioned media were identified by Western ligand and antibody analyses. IGFBP-4 was secreted by all cell lines and IGFBP-2 from the COLO205 and SW620 cells lines but not the HT29 cells. No IGFBP-3 was secreted b any of the cell lines but IGFBP-3 was found in the cell layer ir all of the cell lines. When endogenous secreted IGFBPs were removed, cell lines were consistently more sensitive to IGF-I than des(1,3)IGF-I suggesting that IGFBP-3 associated with the cell layer enhances responses to IGF-I. This is in contrast to the effects of the secreted IGFBPs. Differential modulating actions of IGFBPs may be important in regulating colon cell

turnover.

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1: Endocrinology 1997 May;138(5):2021-32

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Up-regulation of insulin/insulin-like growth factor-I hybrid receptors during differentiation o HT29-D4 human colonic carcinoma cells.

Garrouste FL, Remacle-Bonnet MM, Lehmann MM, Marvaldi JL, Pommier GJ.

Unite Interactions entre Systemes Proteiques et Differenciation dans la Cellule Tumorale, CNRS URA 1924, Faculte de Medecine, Marseille, France.

To assess the autocrine function of insulin-like growth factor II (IGF-II) in the balance of proliferation and differentiation i HT29-D4 human colonic cancer cells, we studied the expression of IGF-I receptors (IGF-IR) and insulin receptors (IR) in relation to the state of cell differentiation. IGF-IR and IR were expressed in both undifferentiated and enterocyte-like differentiated HT29-D4 cells. IGF-IR had two isoforms with a 97-kDa and a 102-kDa beta-subunit. In addition, HT29-D4 cells expressed hybrid receptors (HR) formed by th association of two alphabeta heterodimers from both IR and IGF-IR. HR were evidenced through 1) inhibition of IGF-I binding by the B6 anti-IR antibody and 2) immunoprecipitation with the alpha-IR3 anti-IGF-IR antibody which revealed an additional 95-kDa IR beta-subunit that disappeared when the heterotetrameric receptor was dissociated by disulfide reduction into alphabeta heterodimer before immunoprecipitation. Like IGF-IR, HR had a high affinity for IGF-I (Kd, approximately 1.5 nM), but did not bin insulin significantly; the latter interacted with the native IR only (Kd, approximately 4 nM). In the differentiated HT29-D4 cell monolayer, all receptor species were strongly polarized

(>97%) toward the basolateral membrane. Moreover, HT29-D4 cell differentiation was accompanied by an approximately 2-fold increase in the number of IR, whereas the number of IGF-I-binding sites was unaltered. However, in differentiated HT29-D4 cells, approximately 55% of the latter were involve in HR vs. approximately 20% in undifferentiated HT29-D4 cells. Thus, HT29-D4 cell differentiation is characterized by a up-regulation (approximately 3-fold) of the level of HR coupled to a down-regulation (approximately 40%) of the level of native tetrameric IGF-IR. Alterations were induced early during the cell differentiation process, i.e. 5 days postconfluence, and remained unchanged for at least 21 day: Taken together, these results suggest that the IGF-II autocrine loop in HT29-D4 cells may trigger distinct signaling pathways if it activates native IGF-IR, which predominate in undifferentiated cells, or if it activates HR, which are up-regulated in differentiated cells.

PMID: 9112401 [PubMed - indexed for MEDLINE]

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1: Eur J Cancer 1995;31A(1):50-5 Related Articles, Books, LinkC

Type I insulin-like growth factor receptors in human colorectal cancer.

Adenis A, Peyrat JP, Hecquet B, Delobelle A, Depadt G, Quandalle P, Bonneterre J, Demaille A.

Departement d'Oncologie Medicale, Centre Oscar Lambret, Lille, France.

Type I insulin-like growth factor (IGF) receptors have been recently characterised in human colorectal cancers. The aim this study was to determine whether type I IGF receptor concentration may be related to prognostic variables in colorectal cancers. Saturation experiments with [125I]IGF-I were performed on membrane preparations of 46 frozen specimens (20 tumours, 26 controls) and analysed according to the Scatchard method. In all the studied cases, we found a single class of high affinity binding sites in both normal and malignant colorectal tissues (median 0.17 and 0.15 nmol/l, respectively). Using paired analysis, we found no significant difference in terms of type I IGF receptor concentration between malignant and normal colorectal tissues. There was also no relationship between type I IGF receptors and any of the tumour characteristics studied. This study does not support a critical role of the type I IGF receptors in the clinica management of colorectal cancers.

PMID: 7695979 [PubMed - indexed for MEDLINE]

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1: Cell Growth Differ 1994 Oct;5(10):1085-91

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Expression of insulin-like growth factor (IGF)-II and IGF-I receptor during proliferation and differentiation of CaCo-2 human colon carcinoma cells.

Zarrilli R, Pignata S, Romano M, Gravina A, Casola S, Bruni CB, Acquaviva AM.

Centro di Endocrinologia ed Oncologia Sperimentale del C.N.R., Dipartimento di Biologia e Patologia Cellulare e Molecolare L. Califano,Facolta de Medicina e Chirurgia, Universita di Napoli Federico II, Italy.

We have studied the expression of insulin-like growth factor type II (IGF-II) and its autocrine role during the proliferation and differentiation of the CaCo-2 colon carcinoma cell line. IGF-II RNA levels were high in proliferating cells and decreased by more than 10-fold when cells ceased to proliferate and differentiated. Immunoreactive IGF-II protein was high in the conditioned media of proliferating cells and decreased 20-fold in the media of differentiated cells. Reduce IGF-II expression was associated with a decrease in IGF-I receptor number that was high in proliferating cells (approximately 80,000 binding sites/cell) and reduced by 4-fold in differentiated cells. Exogenously added IGF-II was abl to stimulate proliferation of serum-deprived cells in a dose-dependent fashion. IGF-II acted through the IGF-I receptor, since both basal and IGF-II-stimulated cell proliferation was inhibited by the monoclonal antibody alpha-IR3, which blocks the binding sites of the IGF-I receptor. The inhibition of CaCc 2 basal cell growth by the alpha-IR3 antibody suggests that IGF-II may act as an autocrine growth factor for these cells.

PMID: 7848910 [PubMed - indexed for MEDLINE]

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